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ANGIOIMMUNOBLASTIC T-CELL LYMPHOMA: HISTOLOGIC, IMMUNOPHENOTYPIC AND MOLECULAR GENETIC CHARACTERISATION OF THE DISEASE

A thesis submitted for the Degree of Doctor of Philosophy in the Faculty of Clinical Sciences of the University of London

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Abstract

Angioimmunoblastic T-cell lymphoma (AITL), although initially thought to be an atypical reactive process - angioimmunoblastic lymphadenopathy with dysproteinaemia (AILD), has since been proved to be a T-cell lymphoma and is categorised as a subtype of peripheral T-cell lymphoma. It is an aggressive disease, in which the biology is poorly understood. Defining histological criteria apply only to typical examples, while many features overlap with reactive conditions and other lymphomas.

In this thesis, CD10 was investigated as a possible phenotypic marker of AITL. As the neoplastic cells comprise the minority, individual CD10 positive lymphoid cells were microdissected for molecular genetic analysis. The results showed that the neoplastic T-cells in AITL expressed CD10, thus providing a marker to identify the neoplastic T-cell. It was also shown that in the assessment of nodal peripheral T-cell lymphomas, CD10 expression appears to be a sensitive and specific marker for AITL. CD10 expression was maintained in most extranodal sites of involvement, and correlated well with the presence of a follicular dendritic cell meshwork.

AITL showed 3 overlapping histological patterns, depending on the presence of hyperplastic follicles (pattern I), regressed follicles (pattern II) or absence of identifiable follicles (pattern III). Histologic progression was studied in consecutive biopsies and it was shown that pattern I represented early lymph node involvement, which progressed to pattern III with disease progression. Furthermore, when AITL was complicated by a "large cell lymphoma" it was usually an EBV-associated diffuse large B-cell lymphoma. EBV-associated B-cell proliferations complicated 25% of cases.

Using CD10 as a phenotypic marker of the neoplastic cell it was shown that the latter expressed CXCL13 and BCL-6 consistent with a germinal centre T-cell origin.

Finally, the correlation between EBV-load and histological patterns was studied and the presence of HHV-8 investigated. A high EBV load was mainly observed in pattern III histology, a feature that probably parallels the increasing degree of immune suppression. HHV-8 infection was not found to be a feature of AITL.

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Abbreviations

AILD	Angioimmunoblastic lymphadenopathy with dysproteinaemia
AITL	Angioimmunoblastic T-cell lymphoma
ALCL	Anaplastic large cell lymphoma
ALK	Anaplastic lymphoma kinase
ATLL	Adult T-cell leukaemia/lymphoma
B2M	B2 microglobulin
BCA-1	B-cell chemoattractant-1
BCIP	5-Bromo-4-Chloro-3-Indolyl Phosphate
C	Constant
C-ALCL	Cutaneous anaplastic large cell lymphoma
CD	Cluster designation
cFLIP	Cellular flice-like inhibitory protein
CHL	Classical Hodgkin lymphoma
CHOP	Cyclophosphamide, Doxorubicin, Oncovin, Prednisone
D	Diversity
DAB	Diamino benzidine
DLBCL	Diffuse large B-cell lymphoma
DDW	Double distilled water
EBER	Epstein Barr virus encoded RNA
EBNA	Epstein Barr virus nuclear antigen
EBV	Epstein Barr virus
EDTA	Ethylene diamine tetra-acetic acid
ETTL	Enteropathy-type T-cell lymphoma
FasL	Fas ligand

FDC	Follicular dendritic cell
FR3	Frame work 3 region
GC	Germinal centre
H&E	Haematoxylin and eosin
HEV	High endothelial venules
HHV-6	Human Herpes virus 6
HHV-8	Human Herpes virus 8
HIV	Human immunodeficiency virus
HTLV-1	Human T-cell lymphoma virus-1
IF γ	Inteferron γ
Ig	Immunoglobulin
IgH	Immunoglobulin heavy chain
IL	Interleukin
ILSG	International lymphoma study group
ISH	In situ hybridisation
J	Joining
LB	Luria Bertani
LMP	Latent membrane protein
LyP	Lymphomatoid papulosis
MF	Mycosis fungoides
MHC	Major histocompatibility complex
MUM-1	Multiple myeloma oncogene-1
NBT	Nitroblue tetrazolium chloride
NHL	Non-Hodgkin lymphoma
NK	Natural killer

NLPHL	Nodular lymphocyte predominant Hodgkin lymphoma
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PTLD	Post-transplant lympho-proliferative disorder
PTL	Peripheral T-cell lymphoma
PTLu	Peripheral T-cell lymphoma, unspecified
REAL	Revised European-American classification
RGS	Regulator of G protein signaling
SDS	Sodium dodecyl sulphate
SS	Sezary syndrome
SSC	Sodium chloride sodium citrate
SSCP	Single stranded conformational polymorphism
TBE	Tris Borate EDTA
TBS	Tris buffered saline
TCR	T-cell receptor
TdT	Terminal deoxynucleotidyl transferase
Th1	T-helper-1
Th2	T-helper-2
TIA-1	T-cell intracellular antigen-1
T-LGL	T-cell large granular cell leukaemia
T-PLL	T-cell prolymphocytic leukaemia
V	Variable
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VLA	Very late activation antigen

WHO	World Health Organization
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

Chapter 1

INTRODUCTION

1.1 LYMPHOMA

1.1.1 Definition and Classification

Lymphoma is defined as a neoplastic proliferation of lymphoid cells. Similar to other neoplasms, lymphoma evolves from a monoclonal proliferation in most instances (Medeiros LJ *et al*, 1995). Due to their propensity for dissemination, all lymphoid neoplasms are considered malignant or potentially malignant (Jaffe, 1995). In terms of behaviour, some are aggressive from the outset while others behave indolently for long periods, but may transform over time into more aggressive, overtly malignant tumours (Jaffe, 1995).

Classification of lymphomas has been at the center of controversy for many decades. Over the years, the schemes that emerged reflected the knowledge (or lack of knowledge) of the normal immune system at the time. However, the overall aim of all of these systems has been to stratify lymphoid neoplasms into reproducible categories that would also be acceptable to clinicians, i.e. have prognostic relevance. The Rappaport classification (1966) and the subsequent modified Rappaport classification for non-Hodgkin lymphoma (NHL) and the Lukes-Butler classification for Hodgkin lymphoma as modified at the Rye conference (1966) were based on the patterns of growth and the cytologic characteristics of the neoplastic elements (Lukes & Butler, 1966;Rappaport, 1966). The subsequent Kiel (Lennert's) classification (1974) and the Lukes-Collins classification (1974), although based on immunological concepts were also primarily morphological, as was the unifying classification scheme developed at the time, the

Working Formulation for Clinical Usage (1982;Gerard-Marchant *et al*, 1974;Lennert *et al*, 1975;Lukes & Collins, 1974). With the advent of immunophenotyping, cytogenetic studies and clonality analysis by southern blotting and polymerase chain reaction (PCR), it became clear that immunological and genetic approaches were essential to the recognition of individual disease (clinical) entities. The "Revised European-American Classification of Lymphoid neoplasms" (REAL) classification, published by the International Lymphoma Study Group (ILSG) in 1994, a consensus list of lymphoid neoplasms uses morphology, immunophenotype, genetic features and clinical features to define a disease entity. The World Health Organization (WHO) classification is based on the principles defined in the REAL classification, but also incorporates input from additional experts in order to update and broaden the consensus on lymphoid neoplasms, and extends the principles of disease definition and consensus building to the classification of myeloid neoplasms, myelodysplastic syndromes, and mast cell and histiocytic/dendritic-cell neoplasms (Harris *et al*, 1994;Harris *et al*, 2000;Harris *et al*, 2001a). The WHO classification of haematological tumours, thus stratifies neoplasms primarily according to lineage, and within each category, distinct entities are defined according to a combination of morphology, immunophenotype, genetic features and clinical syndromes. For each neoplasm, a cell of origin, representing the stage of differentiation of the tumour cells as seen in the tissues, and not necessarily the cell in which the initial transforming event occurred, is postulated. The classification recognizes three major categories of lymphoid neoplasms: B-cell neoplasms, T-cell and NK-cell neoplasms and Hodgkin Lymphoma. The B-cell neoplasms and T-cell and NK-cell neoplasms are in turn subcategorized into precursor neoplasms, mature neoplasms and proliferations of uncertain malignant potential (Table 1.1) (Harris *et al*, 2001a).

Table 1.1 Section of WHO Classification that stratifies tumours of lymphoid tissues

B-CELL NEOPLASMS
Precursor B-cell neoplasms
Precursor B lymphoblastic leukaemia / lymphoma
Mature B-cell neoplasms
Chronic lymphocytic leukaemia / small lymphocytic lymphoma
B-prolymphocytic leukaemia
Lymphoplasmacytic lymphoma
Splenic marginal zone lymphoma
Hairy cell leukaemia
Plasma cell myeloma
Solitary plasmacytoma of bone
Extraosseous plasmacytoma
Extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma)
Nodal marginal zone B-cell lymphoma
Follicular lymphoma
Mantle cell lymphoma
Diffuse large B-cell lymphoma
Mediastinal (thymic) large B-cell lymphoma
Intravascular large B-cell lymphoma
Primary effusion lymphoma
Burkitt lymphoma / leukaemia
B-cell proliferations of uncertain malignant potential
Lymphomatoid granulomatosis
Post-transplant lymphoproliferative disorder, polymorphic
T-CELL AND NK-CELL NEOPLASMS
Precursor T-cell neoplasms
Precursor T lymphoblastic leukaemia / lymphoma
Blastic NK cell lymphoma
Mature T-cell and NK cell neoplasms
T-cell prolymphocytic leukaemia
T-cell large granular lymphocytic leukaemia
Aggressive NK cell leukaemia
Adult T-cell leukaemia/lymphoma
Enteropathy-type T-cell lymphoma
Hepatosplenic T-cell lymphoma
Subcutaneous panniculitis-like T-cell lymphoma
Mycosis fungoides
Sezary syndrome
Primary cutaneous anaplastic large cell lymphoma
Peripheral T-cell lymphoma, unspecified
Angioimmunoblastic T-cell lymphoma
Anaplastic large cell lymphoma
T-cell proliferations of uncertain malignant potential
Lymphomatoid papulosis
HODGKIN LYMPHOMA
Nodular lymphocyte predominant Hodgkin lymphoma
Classical Hodgkin lymphoma
Nodular sclerosis classical Hodgkin lymphoma
Lymphocyte-rich classical Hodgkin lymphoma
Mixed cellularity classical Hodgkin lymphoma
Lymphocyte-depleted classical Hodgkin lymphoma

Both lymphomas and leukaemias are included, as both solid and circulating (leukaemic) phases may be present.

Although uncommon, mature T-cell and NK-cell neoplasms include some of the most (clinically) aggressive lymphomas (1982;Coiffier *et al*, 1990;Gisselbrecht *et al*, 1998;Melnyk *et al*, 1997). In the sections that follow, mature T-cell and NK-cell neoplasms will be discussed and angioimmunoblastic T-cell lymphoma (AITL) reviewed in detail. As these neoplasms retain many of the properties of various subsets of normal T-cells it would be appropriate to first briefly review normal T-cell biology, followed by an overview of clonality analysis in lymphoid proliferations.

1.2 T-cell biology

1.2.1 T-cell subsets: phenotypic properties and function

T-cells constitute 50-70% of circulating lymphocytes, and like B-cells harbour diversified, antigen specific receptors on their surface. T-cells identify and bind peptide antigens displayed at the host cell surface by the glycoproteins of the major histocompatibility complex (MHC) (Davis & Bjorkman, 1988;Kay, 1991). Two main subsets of T-cells, α/β T-cells and γ/δ T-cells, distinguished by the expression of α/β or γ/δ T-cell receptor complex, respectively (Bentley & Mariuzza, 1996;Goodman & Lefrancois, 1988). Over 95% of T-cells in the peripheral blood, lymph nodes and spleen are α/β T-cells whereas the γ/δ T-cells, which are not MHC restricted, comprise less than 5% of all normal T-cells and are mainly confined to the splenic red pulp, intestinal epithelium and other epithelial sites (Asarnow *et al*, 1989;Bucy *et al*, 1989;Delves & Roitt, 2000a;Goodman & Lefrancois, 1988;Sciammas *et al*, 1994).

As α/β and γ/δ T-cell receptors are non-covalently linked to the CD3 molecular complex, which contains γ,δ and ε chains, CD3 is expressed on both α/β and γ/δ T-cells (Delves & Roitt, 2000b;Jaffe & Ralfkiaer, 2001b). NK-cells usually express ε chain of CD3 in the cytoplasm, which can be detected by polyclonal antibodies to CD3 (Jaffe & Ralfkiaer, 2001b;Lanier *et al*, 1992a). The α/β T-cells are divided into two subtypes depending on whether they are CD4-positive or CD-8 positive. In normal lymphoid tissues the CD4-positive (helper) T-cells exceed the CD-8 positive (cytotoxic/suppressor) T-cells (Reinherz & Schlossman, 1980). The CD4-positive T-cells recognize peptides at the antigen presenting cell surface in the context of the MHC class II molecules, whereas the CD8 positive T-cells recognize antigen in the context of MHC class I molecules (Delves & Roitt, 2000a). All mature α/β T-cells in addition to CD3, express CD2, CD5 and CD7 (Berney *et al*, 2000;Berney *et al*, 2001;Haynes *et al*, 1988;Reinherz & Schlossman, 1980). The γ/δ T-cells are negative for CD4, CD5 and usually for CD8, although a subpopulation has been shown to express the latter. NK-cells show some characteristics of cytotoxic T-cells and can express CD2, CD7, CD8, CD56 and CD57, antigens that may also be expressed by T-cells (Berney *et al*, 2000;Jaffe & Ralfkiaer, 2001b;Lanier *et al*, 1992b). With regard to leucocyte common antigen (LCA) or CD45 expression by mature T-cells, naïve T-cells express the 205-kd isoform that is recognized by the anti-CD45, CD45RA (CD45RA positive) but not by CD45RO (CD45RO negative), while in contrast the memory T-cells express the 180-kd isoform that is CD45RA negative but CD45RO positive (Beverley, 1992).

TCR binding in the context of MHC also requires a co-stimulatory signal in order to trigger a T-cell response to antigen. This co-stimulatory signal is provided by CD28, which binds to CD80 (B7-1) or CD86 (B7-2) expressed by antigen presenting cells (June

et al, 1994). Upon activation in secondary lymphoid tissues naïve CD4 positive T-cells undergo clonal expansion and differentiation to become memory or effector cells. According to their effector function and cytokine secretion profile, memory and effector CD4 positive T-cells, are divided into nonpolarised T-cells, T-helper 1 (Th1), T helper 2 (Th2) and regulatory T-cells (Kim *et al*, 2001a). Th1 cells secrete interleukin (IL) 2 and interferon γ (IFγ), which promote cellular immunity, whereas the Th2 cells induce humoral immunity by the production of cytokines IL4, 5, 6 and 10 (Delves & Roitt, 2000b). According to their chemokine receptor profile, effector and memory T-cells may also be classified into "central memory cells" that are CXCR5+, CCR7+ and "effector memory cells" that are predominantly CCR7- (Kim *et al*, 2001b;Kim *et al*, 2001a;Sallusto *et al*, 1999). Th1 and Th2 cells are derived from the latter. CCR5 and CXCR3 chemokine receptors are markers of Th1 cells, while CXCR4, CCR3 and CCR4 are preferentially expressed by Th2 cells (Bonecchi *et al*, 1998;Kim *et al*, 2001a;Weng *et al*, 2003). Germinal centres (GCs) have a population of CD4 positive, helper T-cells that express CD57 and also express CXCR5 but not CCR7 (Chtanova *et al*, 2004;Kim *et al*, 2001b;Velardi *et al*, 1988). They are distinct from Th1 and Th2 cells both in terms of effector functions and cytokine secretion profile.

1.2.2 T-cell ontogeny

Progenitor cells produced in the bone marrow are processed in thymus, due to their interaction with cytokines and thymic stroma. The pluripotent cells that arrive in the thymus give rise to the earliest definitive T-cells, the pro-thymocytes that express CD2 and CD44 (Schattner EJ & Casali P, 2001). The latter, located in the subcapsular region of the thymic cortex have their TCR-β gene in germ line configuration and can thus

develop into either α/β or γ/δ subsets. Maturation within the thymus and movement from the thymic cortex to the medulla results in the stepwise acquisition/loss of surface antigens (Figure 1.1), TCR gene rearrangement (see below) and T-cell selection, the latter depending on their affinity for MHC binding and lack of auto-reactivity (Delves & Roitt, 2000a).

1.2.3 T-cell receptor genes

The T-cell receptor (TCR) is the means by which T-cells recognize and interact with processed antigen. The function of this receptor involves interaction with a multitude of foreign molecules. To meet this challenge, complex processes of gene rearrangement have evolved to provide the required diversity (Delves & Roitt, 2000a). There are 4 TCR proteins, α, β, γ and δ, which give rise to 2 types of heterodimeric T-cell receptors, the α/β and γ/δ heterodimers (Delves & Roitt, 2000a).

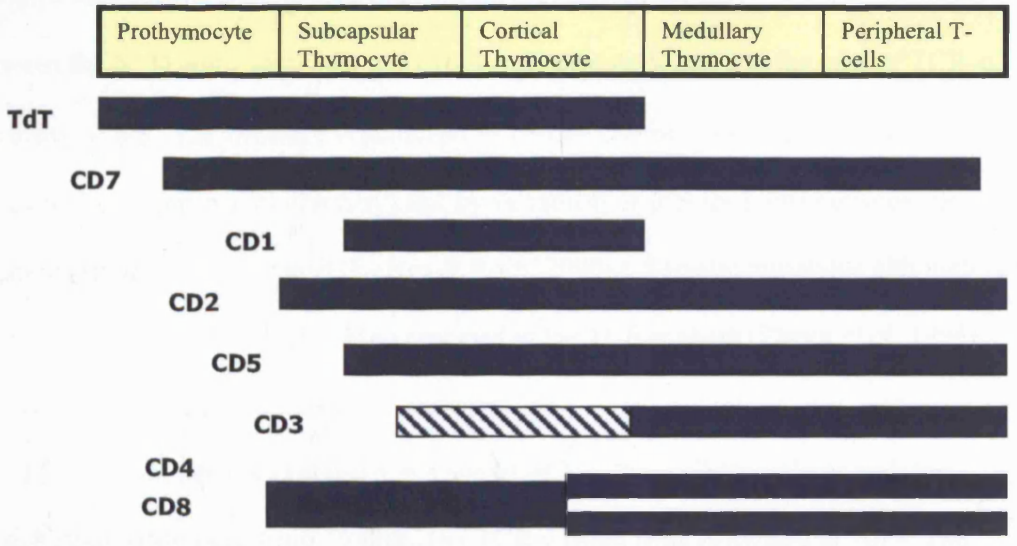

Figure 1.1 Stepwise acquisition/loss of surface antigens during T-cell ontogeny
(adapted from (Stetler-Stevenson *et al*, 1995)

All 4 TCR genes are composed of many non-identical gene segments that must rearrange into a functional unit prior to transcription and production of the protein. The human TCR-α chain and TCR-β chain genes are located at 14q11(Croce *et al*, 1985) and 7q34 (Caccia *et al*, 1984), respectively. Both genes are composed of multiple segments in the germline that rearrange to form functional variable regions that combine with constant (C) regions. The TCR-α chain gene has 50-100 variable (V) segments, 50-100 joining (J) segments and 1 C region (Pan *et al*, 2001). The TCR-β gene has 75-100 V segments along with 2 arrays composed of a single D (diversity) segment, 13 J segments and 2 C regions (Figure 1.2) (Pan *et al*, 2001). In the case of TCR-β, rearrangement of the gene involves excision of DNA between the D and J clusters resulting in D-J joining followed by a second excision between the D and V cluster resulting in V-DJ joining (Figure 1.2) (Pan *et al*, 2001). The VDJ unit becomes the coding sequence for the variable part of the TCR protein, which is expressed in conjunction with C region exons. Variable numbers of untemplated nucleotides (N regions) are inserted at the junctions between the V, D and J segments in TCR-β and between V and J in the case of TCR-α. Diversity of the TCR structure is achieved by unique combinations of different V, D and J regions (recombinatorial diversity) and by variability in precise joints between the segments (junctional diversity) (Delves & Roitt, 2000a). Somatic mutations although not a well recognized feature, have been reported in the TCR-α chain (Zheng *et al*, 1994) and TCR- β chain gene (Cheynier *et al*, 1998).

The TCR-γ chain gene is expressed in a subset of T-cells as a heterodimer with the TCR-δ chain (Delves & Roitt, 2000a).The TCR-γ chain gene is located at 7p15. The locus contains 14 V segments, which can be divided into 6 families by sequence homology, 5 J segments in 2 clusters and 2 C regions (Figure 1.2) (Pan *et al*, 2001). N

regions are found at the V-J junctions, but no evidence of somatic hypermutation has been reported. The delta chain gene is situated at 14q11 within the TCR-α chain gene (Chien *et al*, 1987;Pan *et al*, 2001). It is composed of at least 10 V segments, 3 D segments, 3 J segments and 1 C region. Variable N regions are found at the V-D and D-J junctions, but somatic mutations have not been reported (Pan *et al*, 2001). Rearrangements of the γ, δ and β genes occur simultaneously, prior to rearrangements of the α gene. Therefore mature T-cells of either α/β or γ/δ type, have rearranged γ and δ TCR genes. (Schattner EJ & Casali P, 2001)

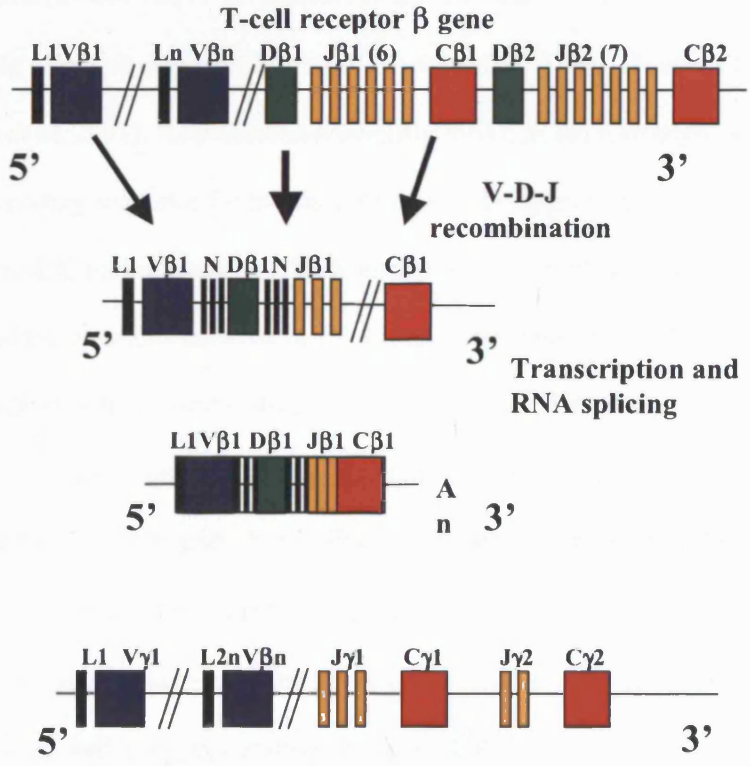

Figure 1.2 Diagramatic representation of T-cell receptor gene rearrangement

As B-cell proliferations may be present in association with mature T-cell neoplasms immunoglobulin (Ig) gene rearrangement will also be discussed briefly at this point.

1.2.3.1 Immunoglobulin gene rearrangement in B-cells

Immunoglobulin molecules are composed of two identical heavy and two identical light chain proteins. Each B-cell expresses a heavy chain in combination with either a κ or λ molecule (Delves & Roitt, 2000a). The heavy chain, κ and λ undergo somatic gene rearrangement. The gene encoding the human heavy chain gene is located onchromosome 14 at q32 (Hobart *et al*, 1981), while the two light chain genes are located at 2p12 (κ) and 22q11 (λ) (Hobart *et al*, 1981;Pan *et al*, 2001;Vasicek & Leder, 1990). The Ig heavy chain gene consists of clusters of V (>100), D (about 30) and J (9) regions (Pan *et al*, 2001). As described previously for TCR, the rearranged VDJ unit becomes the coding sequence for the variable part of the Ig protein, which is expressed in conjunction with C region exons. Variable numbers of untemplated nucleotides (N regions) are inserted at the junctions between the V, D and J segments. As for TCR, diversity of Ig structure is achieved by recombinatorial diversity, junctional diversity, but in addition also importantly by somatic point mutations. The latter are introduced into the variable regions of fully assembled genes as part of an "affinity maturation" process in follicle centres in which antibody binding is enhanced during the immune response. The κ and λ light chain genes have equivalent structures to the heavy chain gene and also undergo somatic rearrangement as well as hypermutation (Pan *et al*, 2001).

1.3 Clonality analysis in the evaluation of lymphoid proliferations

The discovery that the genes for the immune recognition molecules (the Igs and T-cell receptors) in lymphocytes undergo somatic rearrangements that are unique to a given lymphocyte and its progeny, has provided a means for clonality analysis in lymphoproliferative disease of both B- and T-cell lineages. The genes encoding these molecules are useful markers of T and B-cell clonality and are used as targets for detection of tumour clones using Southern blot and PCR.

1.3.1 Southern blot analysis

For many years, Southern blot analysis has been the gold standard technique for clonality analysis in lymphoid proliferations. It is used to determine gene structure by restriction fragment size analysis (Southern, 1975). The technique involves the use of a probe (most frequently, a J segment probe) which hybridises to the variable region of TCR or Ig heavy or light chain genes and detects a germline band of predictable size in DNA from non-T-cells (in the case of TCR) or non-B-cells (in the case of Ig), which do not carry rearranged antigen receptor genes. Rearrangement of the TCR and Ig genes generate restriction fragments of different sizes. Monoclonal populations carry identical antigen receptor gene rearrangements, and therefore Southern blot analysis of tumour DNA results in a hybridising fragment (or two fragments if both alleles are rearranged) of different size to the germline. Typical clinical samples, containing both tumour and other cells, yield both a germline band and novel rearranged fragments. Tumour samples must contain at least 1-2.5% of tumour cells to allow a confident interpretation of

monoclonality (Cleary *et al*, 1984). In the evaluation of T-cell proliferations, the TCR-α, TCR-β, TCR-γ and TCR-δ genes have all been targeted, although the TCR-β and TCR-γ have been the most frequently used (Davey *et al*, 1986;O'Connor *et al*, 1985;Yokota *et al*, 1991). Studies of the TCR-α and TCR-δ chain genes have been less frequent because blotting of the TCR-α gene results in complex germline patterns and the TCR-δ chain gene is deleted by rearrangement of the TCR-α chain gene and therefore DNA extracted from such clones does not hybridise with the probe (Cossman & Uppenkamp, 1988;Hockett *et al*, 1988). Due to limitations to its application, although highly reliable, Southern blotting is increasingly replaced by PCR. The method is time consuming, expensive, requires radioactive isotopes, is technically demanding, and has a limited sensitivity of 5-10%. However the most important limitation of Southern blot analysis in diagnostic histopathology is the requirement for high quality DNA, i.e. fresh or frozen tissue samples, only available in a minority of cases. Attempts have been made to use DNA extracted from formalin fixed, paraffin embedded tissues but the results have been unreliable (Warford *et al*, 1988).

1.3.2 Polymerase chain reaction (PCR)

PCR was first described in practice by Saiki and co-workers (Saiki *et al*, 1985) who amplified specific β-globin gene fragments from genomic DNA. PCR has since become one of the most widely used analytical tools in molecular genetics as it permits rapid in vitro amplification of specific DNA sequences from minute, complex and even degraded nucleic acid sources and is technically simple. Amplified DNA can be readily examined for structural changes. These features are of particular importance in histopathology because PCR allows molecular genetic analysis of formalin fixed, paraffin embedded

biopsies in which the nucleic acid is highly degraded and present in small quantities. (Diss & Pan L, 1997;Goudie, 1989;Pan *et al*, 1995;Quirke & Taylor, 1989). PCR is a single tube reaction which relies on a thermostable DNA polymerase (usually Taq polymerase) to duplicate DNA sequences from a template using oligonucleotide primers which flank, and specifically bind to, the DNA region of interest (Saiki *et al*, 1988). Amplification is achieved by multiple repetition (30-40 times) of cycles of temperature changes, in which each cycle is composed of three stages. These are (1) denaturation of template at 90-95°C, (2) annealing of primers to their complementary sequences at each end of the target gene fragment at 50-60°C, (3) new strand polymerisation by extension from the primers at 72°C. Primer extension occurs in a 5' to 3' direction only, so only DNA between the primers is amplified. The consequence of a single cycle of PCR is a doubling of specific double stranded DNA sequence. Thus specific target fragments increase exponentially upon repetition of the cycle (Pan *et al*, 1995). Computer controlled heating blocks allow automated temperature cycling and precise control of reaction conditions.

Increased sensitivity and specificity can be achieved using nested PCR, which involves two rounds of amplification (Wan *et al*, 1990). An initial amplification of the test DNA using "outer primers" is followed by amplification of an aliquot from the first round of PCR using "inner primers". Typically 20-30 cycles are used in each round.

PCR products can be analysed using agarose (AGE) or polyacrylamide gel electrophoresis (PAGE) (Pan *et al*, 1995). This allows confirmation of the presence of target template DNA, and shows the size of the DNA fragment under study. Any gross changes such as deletions or insertions can be identified. Restriction analysis of PCR products can be carried out to search for mutations or polymorphisms. For sequence

related analysis, temperature gradient or denaturing gradient gel electrophoresis (DGGE) (Bourguin *et al*, 1990), or single strand conformational polymorphism (SSCP) analysis may be employed (Orita *et al*, 1989).

For the purpose of clonality analysis, primers are designed so that they flank known rearranged segments of Ig/TCR genes. Although in fresh/frozen tissue the distance between primers may approach 500 base pairs (bp), in formalin-fixed tissues this should be less than 300 bp. Due to the limited number of primers required, Ig heavy chain (IgH) and TCR-γ gene rearrangements were commonly used for B- and T-cell clonality studies respectively. As TCR-γ gene rearrangements are a feature of all T-cell types, including those expressing TCR-α/β chains, the TCR-γ gene has been considered a useful target for clonality detection in all T-cell proliferations (Diss *et al*, 1995). Although PCR as a technique for clonality detection has clear advantages, false negative results due to improper primer annealing and difficulties in discriminating monoclonal from polyclonal gene rearrangements are known problems. This prompted the initiation of BIOMED-2 concerted action BMH4-CT98-393 with the aim of overcoming these shortcomings by careful selection of PCR targets, the use of multiple primer sets designed for multiplex reactions and standardized protocols developed on pre-existing experience from previous European collaborative studies (van Dongen *et al*, 2003). In the BIOMED-2 protocol for Ig gene rearrangements, IgH (complete VH-JH rearrangements and incomplete DH-JH rearrangements), Igκ (Vκ-Jκ and κ-deletion rearrangements) and Igλ (Vλ-Jλ) are all targeted in order to include GC or post-GC derived B-cell proliferations with somatic hypermutations that may otherwise be missed. For TCR-gene rearrangements, targets include both TCR-β and TCR-γ genes, while TCR-δ primers are added only for precursor malignancies and γδ T-cell proliferations

(van Dongen *et al*, 2003). Due its complexity, with numerous V and J gene segments TCR-α is not included. However this does not appear to be a problem as all T-cell neoplasms with TCR-α gene rearrangements contain TCR-β gene rearrangements and TCR-γ gene rearrangements as well (van Dongen *et al*, 2003).

1.4 Mature T-cell and NK-cell neoplasms

1.4.1 Definition

Mature T-cell neoplasms are derived from mature /post-thymic T-cells. As NK-cells are closely related and share some characterististics with T-cells, NK-neoplasms and mature T-cell neoplasms are categorized together (Jaffe & Ralfkiaer, 2001b). WHO histological classification of mature T-cell and NK-cell neoplasms is presented in Table 1.1.

1.4.2 Epidemiology

Mature T-cell and NK-cell neoplasms account for only approximately 12% of all NHL, worldwide. The incidence of individual subtypes is summarized in Table 1.2 (1997) Mature T-cell and NK-cell neoplasms show a geographic variation in incidence, being more common in Asia (2000). This is partly accounted for by the increased incidence of Human T-cell lymphoma virus-1 (HTLV-1) associated adult T-cell leukaemia/lymphoma (ATLL) in endemic regions in Japan and the apparent racial predisposition of Asians for the development of Epstein Barr virus (EBV) associated nasal and nasal type NK/T-cell lymphomas and aggressive NK/T-cell leukaemia (2000). Mature T-cell lymphomas or peripheral T-cell lymphomas (PTLs), generally affect older people (>50-years of age) and males are more frequently affected than females (Savage

et al, 2004). Anaplastic large cell lymphoma (ALCL), however, is an exception in that it tends to affect children and young adults (Stein *et al*, 2000). The occurrence of mature T- and NK-cell neoplasms in patients with a prior history of a B-cell lymphoma, or presenting concurrent with or prior to the development of a B-cell lymphoma has been reported (Abruzzo *et al*, 1993;Xu *et al*, 2002a).

1.4.3 Immunophenotype and cytokine secretion profile

Although mature T-cell neoplasms (PTLs) show the immunophenotypic features of mature post-thymic T-cells, they may display an abnormal or aberrant phenotype such as loss of CD5 expression or co-expression or loss of CD4 and CD8 in α/β T-cells, a feature which when present, is of use in distinguishing lymphoma from a reactive T-cell proliferation (Stetler-Stevenson *et al*, 1995).

The predominance of α/β T-cells in normal T-cells, is also reflected in mature T-cell lymphomas where α/β T-cell lymphomas far exceed the rare γ/δ T-cell lymphomas (Table 1.2). Although cytokine secretion profiles do not determine subtypes of T-cell lymphomas, cytokine production by neoplastic cells plays an important role in the clinical manifestations of T-cell lymphomas. For example, the haemophagocytic syndrome associated with PTLs is attributed to the secretion of cytokines (Teruya-Feldstein *et al*, 1999). Studies show that cytokine or more specifically chemokine receptors specific for Th1 or Th2 cells are expressed in more or less non-overlapping subsets of PTLs. For example, the chemokine receptor CXCR3, expressed in Th1 T-cells is expressed mainly by some PTL, unspecified (PTLu), and by most AITL, whereas the CCR4 and CXCR4, chemokine receptors, expressed by Th2 cells are a feature of ALK-

positive anaplastic large cell lymphoma (Ishida *et al*, 2004;Jones *et al*, 1999;Ohshima *et al*, 2004;Tsuchiya *et al*, 2004).

Table 1.2 Incidence of peripheral T-cell lymphomas

Diagnosis	% of all NHLs
Peripheral T-cell lymphoma, unspecified	3.7%
Angioimmunoblastic T-cell lymphoma	1.2%
Anaplastic large cell lymphoma	2.4%
Extranodal NK/T-cell lymphoma, nasal type	1.4%
Enteropathy-type T-cell lymphoma	<1%
Hepatosplenic T-cell lymphoma	<1%
Adult T-cell leukaemia/lymphoma	<1%

Data taken from "A clinical evaluation of the International Lymphoma Study Group classification of non-Hodgkin's lymphoma. The Non-Hodgkin's Lymphoma Classification Project" 1997.

1.4.4 Survival

With the exception of anaplastic large cell lymphoma (ALCL), most mature T-cell and NK-cell lymphomas are clinically aggressive with a much poorer response to therapy and a shorter survival than B-cell lymphomas (Gisselbrecht *et al*, 1998;Melnyk *et al*, 1997;Rudiger *et al*, 2002). Although the T-cell phenotype is by itself an independent adverse prognostic factor in non-ALCL, PTL (Gisselbrecht *et al*, 1998), the poor response to therapy may be in part due to the fact that many patients present at an advanced stage of the disease and also that the treatment regimens employed are those developed for B-cell lymphomas, rather than specifically for T-cell lymphomas. This may be due to the rarity of T-cell lymphomas, as a result of which they are less well characterised compared to B-cell lymphomas, and few trials have been restricted to them.

In the following sections leukaemic/disseminated, cutaneous, other extranodal mature T-cell and NK-cell neoplasms and blastic NK-cell lymphoma, a neoplasm of uncertain

lineage and stage of differentiation will be mentioned briefly, followed by a more detailed review of nodal T-cell lymphomas, with special emphasis on AITL (AITL).

1.4.5 Leukaemic/ disseminated mature T-cell and NK-cell neoplasms

1.4.5.1 T-cell prolymphocytic leukaemia (T-PLL)

T-PLL is an aggressive leukaemia comprising small to medium sized "pro-lymphocytes". They express CD2, CD3 and CD7. Although the majority is CD4+, CD8-, they may be CD4+, CD8+ (a feature almost unique to T-PLL) or CD4-, CD8+. Consistent with a mature phenotype they are TdT (terminal deoxynucleotidyl transferase) and CD1a negative. Patients characteristically have disseminated disease with involvement of blood, bone marrow, lymph nodes, liver, spleen and skin (Matutes *et al*, 1991).

1.4.5.2 T-cell large granular lymphocytic leukaemia (T-LGL)

T-LGL is characterised by a persistent increase in the number of large granular lymphocytes in the peripheral blood, without an identifiable cause. Most cases show a CD3+, CD4-, CD8+ phenotype, but rarely CD4+ CD8-, CD4+ CD8+ or CD4-, CD8- may be seen. The disease is often indolent with a minority which may transform to an aggressive PTL (Harris *et al*, 1994;Harris *et al*, 2000).

1.4.5.3 Aggressive NK-cell leukaemia

This disease is an aggressive leukaemia that shows an NK-cell CD2+, surface CD3-, CD3ε+, CD56+ phenotype. CD11b and CD16 may be positive but CD57 is usually

negative. Most cases are EBV positive (Chan *et al*, 2001b;Cheung *et al*, 2003;Imamura *et al*, 1990).

1.4.5.4 Adult T-cell leukaemia/lymphoma (ATLL)

ATLL is caused by the HTLV-1 and characterised by systemic disease with involvement of lymph nodes, blood, bone marrow, spleen, liver, skin, lung, gastro-intestinal tract and central nervous system. The neoplastic cells are medium to large in size and show marked nuclear pleomorphism often with polylobated nuclei. The neoplastic cells are CD2+, CD3+, CD5+ but usually CD7-. Most cases are CD4+ CD8-, but rare cases of CD4- CD8+, or CD4+ CD8+ may be seen (Harris *et al*, 1994;Harris *et al*, 2000).

1.4.6 Cutaneous mature T-cell and NK-cell neoplasms

1.4.6.1 Mycosis fungoides (MF)

MF presents in the skin with patches and / or plaques and is characterised by an epidermotropic and dermal infitrate of small to medium-sized cells with "cerebriform" nuclei. The typical phenotype is CD2+, CD3+, CD5+, CD4+ and CD8-. CD7 is often negative, although of limited diagnostic value as this phenomenon may be seen in benign cutaneous lymphoid lesions. Patients with limited disease usually have an excellent prognosis whereas those with more advanced disease have a poorer prognosis (Harris *et al*, 1994;Harris *et al*, 2000;Willemze *et al*, 1997;Willemze *et al*, 2005).

1.4.6.2 Sezary syndrome (SS)

SS is characterised by erythroderma, lymphadenopathy and neoplastic T-cells (with cerebriform nuclei) in the peripheral blood. Although traditionally regarded as a variant of MF this tumour is usually much more aggressive. The tumour cells are CD2+, CD3+, CD5+ and CD7+/- and most cases are CD4+ and CD8- (Harris *et al*, 1994;Harris *et al*, 2000;Willemze *et al*, 1997;Willemze *et al*, 2005).

1.4.6.3 Primary cutaneous CD30+ T-cell lymphoproliferative disorders

There are 3 primary cutaneous CD30+ lympho-proliferative disorders (Harris *et al*, 1994;Harris *et al*, 2000;Willemze *et al*, 1997;Willemze *et al*, 2005). These are:

1. Primary cutaneous anaplastic large cell lymphoma (C-ALCL): C-ALCL is defined as a T-cell lymphoma presenting in the skin and comprising anaplastic lymphoid cells, the majority of which are CD30+. This condition needs to be distinguished from systemic ALCL with secondary cutaneous involvement for which careful staging is required and also from other PTL with CD30 expression. C-ALCL is generally associated with a good prognosis.
2. Lymphomatoid papulosis (LyP): LyP is a chronic recurrent, spontaneously regressing papular skin condition. Morphologically it is characterised by an atypical T-cell infiltrate. The disease has a benign course and is therefore categorised as an atypical lympho-proliferation that can be clonal and sometimes progress to lymphoma.
3. Borderline lesions: These are lesions where there is a discrepancy between the clinical features and the histology, i.e patients with clinical features of LyP but with the histology more in keeping with C-ALCL and vice versa.

1.4.7 Other extranodal mature T-cell and NK-cell neoplasms

1.4.7.1 Extranodal NK/T-cell lymphoma, nasal type

This is a predominantly an extranodal lymphoma that can show varied morphology. As the name implies, the nasal cavity is the commonest site of involvement. The neoplastic infiltrate is often angiocentric with prominent necrosis. EBV is usually present in the clonal episomal form. Most cases are CD56+ (NK-cell phenotype) and rarely CD56- (cytotoxic T-cell phenotype). The disease is most prevalent in Asia and Central and South America. The prognosis of the nasal tumours is variable while those occurring outside the nasal cavity are often highly aggressive (Cheung *et al*, 2003;Harris *et al*, 2000).

1.4.7.2 Enteropathy-type T-cell lymphoma (ETTL)

ETTL is defined as a tumour of intra-epithelial lymphocytes, which usually present following transformation with a tumour composed of large lymphoid cells. There is a clear association with celiac disease and hence the previous term for this disease "Enteropathy-associated T-cell lymphoma" (Isaacson, 1994). The neoplastic cells are CD3+, CD5-, CD7+, CD8 -/+, CD4-, CD103+ and are also positive for cytotoxic granule associated proteins. The disease is one of the most aggressive lymphomas with a poor prognosis (Harris *et al*, 2000;Isaacson *et al*, 2001).

1.4.7.3 Hepatosplenic T-cell lymphoma

This is a rare and aggressive T-cell lymphoma characterised by extranodal disseminated disease. The tumour, derived from cytotoxic T-cells and usually of the γδ T-cell receptor type, shows prominent sinusoidal infiltration of liver, spleen and bone marrow (Belhadj *et al*, 2003;Cooke *et al*, 1996).

1.4.7.4 Subcutaneous panniculitis-like T-cell lymphoma

It is a rare, aggressive form of lymphoma derived from cytotoxic T-cells with a predilection for subcutaneous tissue. The phenotype is usually CD8+ with expression of cytotoxic granule associated proteins. Most cases are derived from αβ cells but a significant minority is γδ positive (Go & Wester, 2004;Kumar *et al*, 1998).

1.4.8 Neoplasm of uncertain lineage and stage of differentiation

1.4.8.1 Blastic NK-cell lymphoma

Blastic NK-cell lymphoma is rare, aggressive and is composed of lymphoblast-like cells showing evidence of commitment to the NK-lineage. Some cases represent precursor NK-cell lymphoblastic lymphoma/leukaemia. The disease tends to involve multiple sites with a predilection for skin and is probably identical to "primary cutaneous CD4+, CD56+ haematodermic neoplasm". The precise lineage of this neoplasm is still not entirely resolved (Koita *et al*, 1997;Petrella *et al*, 2002).

1.4.9 Nodal peripheral T-cell lymphomas

Nodal PTLs include PTLu, anaplastic large cell lymphoma (ALCL) and AITL. PTLu will be discussed in some detail while anaplastic large cell lymphoma will be touched on briefly. The main emphasis of the following review will however be on AITL.

1.4.9.1 Peripheral T-cell lymphoma, unspecified (PTLu)

1.4.9.1.1 *Definition (WHO classification)*

There is a large group of predominantly nodal T-cell lymphomas that cannot be categorized into any of the recognized subtypes of T-cell neoplasms. These are referred to in the WHO classification as PTL with the optional addition of "unspecified", to indicate that they cannot be designated to any of the currently recognized entities. Although various morphological patterns, included as subtypes in other classification systems are described, these are not considered as subtypes by the WHO classification, because of lack of evidence for a clinico-pathological basis for such a distinction (Ralfkiaer *et al*, 2001).

1.4.9.1.2 *Synonyms in previous classification systems*

(1982;Gerard-Marchant *et al*, 1974;Harris *et al*, 1994;Harris *et al*, 2000;Lennert *et al*, 1975;Lukes & Collins, 1974;Ralfkiaer *et al*, 2001):

Lukes-Collins classification: T-immunoblastic lymphoma

Kiel classification: Includes a number of categories - T-zone lymphoma, lympho-epithelioid (Lennert) lymphoma, medium sized, and large cell types, T-immunoblastic lymphoma.

Working formulation: Includes a number of categories – diffuse small cleaved cell, diffuse mixed small and large cell, diffuse large cell and immunoblastic subtypes.

REAL classification: PTLu (provisional categories: large cell, medium-sized cell and mixed large/medium sized cell)

1.4.9.1.3 *Epidemiology*

This category, which includes a heterogeneous group of yet undefined entities, accounts for approximately 3.7% of all lymphomas and for more than half of the PTLs in the West (Table 1.2). PTLu affects mainly adults, although children may also be affected. Males and females are affected equally (1997).

1.4.9.1.4 *Clinical features*

Most patients present at an advanced stage of the disease with constitutional symptoms (B-symptoms). Involvement is predominantly nodal, but any site may be affected, and bone marrow, spleen, liver and extranodal sites including skin are often involved with disseminated disease. Peripheral blood is often involved. Paraneoplastic features such as eosinophilia and haemophagocytic syndrome may occur (1997;Gutierrez *et al*, 2003;Savage *et al*, 2004).

1.4.9.1.5 *Morphology*

In lymph nodes, the architecture is effaced by a diffuse infiltrate, which in most cases shows a predominance of medium to large pleomorphic cells with irregular nuclei, prominent nucleoli and many mitoses. Clear cells and Reed-Sternberg (RS)-like cells may be present. In rare cases, small atypical lymphoid cells may predominate. The background often comprises a polymorphic reactive infiltrate of small lymphocytes, many eosinophils, plasma cells and in some cases, clusters of epithelioid histiocytes. High endothelial venules are often prominent and may show an arborising pattern. There is no increase in FDC meshworks as described in AITL. (see below). Rare

morphological patterns such as the T-zone variant, lympho-epthelioid variant (Lennert lymphoma) are described (Harris *et al*, 1994;Harris *et al*, 2000).

T-zone variant shows an interfollicular growth pattern with preserved or hyperplastic follicles. Neoplastic cells are small to medium sized and do not show marked nuclear atypia. Clusters of clear cells are often a feature and RS-like cells may be present. High endothelial venules are increased. The inflammatory background is prominent and includes eosinophils, plasma cells and epithelioid histiocytes. On morphology and immunophenotyping alone it is difficult to differentiate this variant from reactive hyperplasia without resort to molecular genetic studies (Ralfkiaer *et al*, 2001).

Lympho-epithelioid cell variant (Lennert lymphoma) was first described Karl Lennert in 1968, who regarded this as a mixed entity that included cases of Classical Hodgkin Lymphoma (Kim *et al*, 1980).

This variant shows a diffuse or rarely an interfollicular infiltrate of predominantly small cells with only mild nuclear atypia. It is characterised by numerous small clusters of epithelioid cell histiocytes. Eosinophils and plasma cells are often part of the reactive background. Clear cells may be seen but are reported to be less frequent than in the T-zone variant and AITL. RS-like cells are common, but high endothelial cells are not usually prominent (Patsouris *et al*, 1990;Ralfkiaer *et al*, 2001;Suchi *et al*, 1987).

Not included among the subtypes of PTLu by the WHO classification, but reported as a more recent series are 3 cases of PTL with a follicular growth pattern (de Leval *et al*, 2001). See section 1.4.9.1.6 for immunophenotype of these cases.

1.4.9.1.6 *Immunophenotype*

The immunophenotype reflects a mature post-thymic T-cell phenotype. Aberrant T-cell phenotypes (such as loss of CD5) are common and a useful feature for diagnosis. Most cases are CD4 positive, CD8 negative (Harris *et al*, 1994;Harris *et al*, 2000). Rare nodal PTLu show a cytotoxic phenotype and some of the latter may be associated with CD56 expression (de Bruin *et al*, 1994). When CD30 is expressed by many of the tumour cells it needs to be differentiated from ALCL (see below).

In 3 cases of PTL reported to show a follicular growth pattern – based on FDC meshworks – the tumour cells showed a CD4 positive BCL-6 positive phenotype, with co-expression of CD10 in 2 of the cases (de Leval *et al*, 2001). Epstein Barr virus although absent in neoplastic cells may be present in reactive B-cells. The latter may be RS-like, express CD30, and even on occasion express CD15 mimicking classical Hodgkin lymphoma (CHL) (Quintanilla-Martinez *et al*, 1999).

1.4.9.1.7 *Genetics*

TCR genes show a monoclonal pattern of gene rearrangement in most cases (Theodorou *et al*, 1994). Although many cytogenetic abnormalities have been reported none is consistent or specific. Trisomy 3 has been reported in the lympho-epithelioid cell (Lennert) and T-zone variants (Schlegelberger *et al*, 1994a).

1.4.9.1.8 *Postulated cell of origin*

Peripheral T-cells in various stages of transformation. (Harris *et al*, 1994;Harris *et al*, 2000).

1.4.9.1.9 *Survival and prognosis*

PTLu are aggressive, often responding poorly to therapy. (Gisselbrecht *et al*, 1998;Savage *et al*, 2004). The overall complete remission rate is 40-64% but the 5-year overall survival rate is 35-40%. (Pellatt *et al*, 2002;Savage *et al*, 2004).

1.4.9.2 Anaplastic large cell lymphoma (ALCL)

1.4.9.2.1 *Definition (WHO classification)*

A T-cell lymphoma comprising lymphoid cells that are usually large with abundant cytoplasm and atypical, often horseshoe-shaped nuclei (hallmark cells). The latter are CD30 positive and most cases express cytotoxic granule associated proteins. Most are positive for anaplastic lymphoma kinase (ALK) protein, but ALK negative cases are also included in this category (Delsol *et al*, 2005;Stein *et al*, 2000).

1.4.9.2.2 *Epidemiology and clinical features*

The ALK-positive ALCL occurs mainly in the 1st 3 decades of life, with a male predominance. The ALK negative ALCL generally affects older individuals. Most cases present with disseminated disease (Stein *et al*, 2000).

1.4.9.2.3 *Morphology*

The common variant (70%) comprises large pleomorphic cells many of which are "hallmark" cells. In the lymphohistiocytic variant (10%) the neoplastic cells are intimately admixed with and often hidden by a prominent histiocytic component. However, CD30 positive tumour cells tend to concentrate around blood vessels where they can be identified fairly easily. The small cell variant (5-10%) comprises a

predominance of small to medium sized neoplastic cells, but hallmark cells are always present and are often in the vicinity of blood vessels. This variant may easily be misdiagnosed as PTLu. Others include the giant cell-rich variant and Hodgkin-like variant. Rare variants included the sarcomatoid, "signet ring", neutrophil-rich and eosinophil-rich subtypes (Delsol *et al*, 2005;Stein *et al*, 2000).

1.4.9.2.4 *Immunophenotype*

The neoplastic cells are characterised by CD30 expression. The large cells show strong staining whereas the smaller tumour cells may show weak/negative staining. ALK expression is seen in 53-89% of cases (Stein *et al*, 2000). Most cases are epithelial membrane antigen positive. The majority express one or more T-cell antigens, but due to loss of pan T-cell antigens it may show an apparent "null" cell phenotype. CD3 is negative in most cases, but CD2 and CD4 are often expressed, as are cytotoxic associated antigens T cell intracellular antigen (TIA-1), granzyme B, and or perforin. CD8 is usually negative. The ALK negative ALCL is less well characterised. However, except for ALK expression it shows morphological and immunophenotypic features of ALCL (Stein *et al*, 2000).

1.4.9.2.5 *Genetics*

Approximately 90% of ALCL's show TCR gene rearrangements. ALK expression is due to an abnormality of the ALK locus on chromosome 2. Although many different abnormalities may occur, the commonest is the translocation t(2;5) (p23;35) between the ALK gene on chromosome 2 and the nucleophosmin (NPM) gene on chromosome 5 (Stein *et al*, 2000).

1.4.9.2.6 *Survival and Prognosis*

The most important prognostic indicator is ALK positivity, which is associated with a favourable prognosis. The overall 5-year survival in ALK positive cases is close to 80% whereas the survival in ALK negative cases is approximately 40% (Delsol *et al*, 2005). In fact some studies have shown that the prognosis in ALK-negative cases is similar to PTLu (ten Berge *et al*, 2003). There is an ongoing debate as to whether the ALK-negative cases should be considered a phenotypic variant of ALCL or be categorized as a different entity. The latter is supported by the recent study by Zettl and and co-workers (Zettl *et al*, 2004) where using comparative genomic hybridization (CGH), they found frequent recurrent chromosomal gains and losses in the ALK-negative group. In contrast, the ALK-positive and cutaneous ALCL showed few recurrent chromosomal imbalances.

1.4.9.3 Angioimmunoblastic T-cell lymphoma (AITL)

1.4.9.3.1 *Background*

The disease currently recognized as AITL was first described in the 1970s as a clinical syndrome characterized by generalized lymphadenopathy, hepatosplenomegaly, anaemia and hypergammaglobulinaemia (Frizzera *et al*, 1974;Lennert, 1979;Lukes & Tindle, 1975). The lymph node histology was observed to show a number of distinctive features such as the partial effacement of normal architecture by a polymorphic inflammatory infiltrate, including large blasts and marked vascular proliferation. Based on these histological appearances, the disease was initially referred to by a variety of terms, including immunoblastic lymphadenopathy (Lukes & Tindle, 1975),

lymphogranulomatosis X (Lennert, 1979), and angioimmunoblastic lymphadenopathy with dysproteinaemia (AILD) (Frizzera *et al*, 1974). The latter term was accepted by most investigators and has come to define this clinical syndrome. AILD was initially thought to be an atypical lymphoid hyperplasia, a pre-malignant lesion, with a tendency to develop into a lymphoma rather than a frank neoplasm at onset. With the advent of immunophenotyping and molecular techniques, it became apparent that most cases of AILD contained monoclonal T-cell populations as well as clonal cytogenetic abnormalities, strongly suggesting that the majority of the patients were neoplastic from the onset. However there is still some dispute over whether it represents a de novo lymphoma or whether it is preceded by an atypical reactive process, AILD (Jaffe & Ralfkiaer, 2001a). AITL was included in the Updated Kiel Classification for Lymphomas (Stansfeld *et al*, 1988), and is accepted as a distinct clinicopathological entity in the current WHO classification (Jaffe & Ralfkiaer, 2001a). It is likely that earlier series, published before the availability of immunophenotyping and molecular tests, are diluted by a number of reactive proliferations.

1.4.9.3.2 *Definition (WHO classification)*

AITL is a PTL characterised by systemic disease, a polymorphous infiltrate involving lymph nodes, with a prominent proliferation of high endothelial venules (HEV) and follicular dendritic cells (FDCs) (Jaffe & Ralfkiaer, 2001a).

1.4.9.3.3 *Synonyms in previous classification systems*

(1982;Gerard-Marchant *et al*, 1974;Harris *et al*, 1994;Harris *et al*, 2000;Jaffe & Ralfkiaer, 2001a;Lennert *et al*, 1975;Lukes & Collins, 1974)

Lukes-Collins classification: immunoblastic lymphadenopathy

Kiel classification: AILD-type (Lymphogranulomatosis X) T-cell lymphoma

Working formulation: includes number of categories – diffuse mixed small and large cell, diffuse large cell, immunoblastic and atypical hyperplasia.

REAL classification: angioimmunoblastic T-cell lymphoma

1.4.9.3.4 *Epidemiology*

AITL is a disease of the middle aged and elderly with a peak onset in the 6th and 7th decade of life (median age reported: 53 – 65 years) (Ohsaka *et al*, 1992;Pautier *et al*, 1999;Rudiger *et al*, 2002;Siegert *et al*, 1995;Tobinai *et al*, 1988). There have been rare reports in children in earlier literature (Fiorillo *et al*, 1981;Stensvold *et al*, 1984), showing some overlapping clinical features but no specific morphologic features, no immunophenotypic evidence to demonstrate expansion of the FDC meshwork and no molecular genetic evidence to support a diagnosis of lymphoma, and therefore may not represent examples of AITL. There is no sex predilection for the disease (male to female ratio: 1.3-0.7:1) (Ohsaka *et al*, 1992;Pautier *et al*, 1999;Siegert *et al*, 1995). AITL is reported to account for 1-2% of all NHL, and 5-20% of all PTL (Lu *et al*, 2004;Savage *et al*, 2004). The patients have a wide geographical distribution and have been reported in the Americas, Europe, Asia and Africa (Au *et al*, 2005;Chen *et al*, 2004;Kim *et al*, 2002b;Lopez-Guillermo *et al*, 1998;Montalban *et al*, 1993;Naresh *et al*, 2004;Savage *et al*, 2004;Schetelig *et al*, 2003). One small series suggests that the incidence of AITL may be higher in Hong Kong than Europe (Rudiger *et al*, 2002).

1.4.9.3.5 *Risk factors and aetiology*

No consistent risk factors for the development of the disease have been reported and to date no aetiological agent has been identified. A significant proportion of patients have a history of drug use (Sasaki & Sumida, 2000;Tobinai *et al*, 1988) in particular antibiotics (Batinac *et al*, 2003;Knoops *et al*, 2002;Sasaki & Sumida, 2000). These are more likely to represent drugs prescribed to the patients because of systemic illness clinically mimicking an infectious process, rather than the primary cause of the disease. A number of infectious diseases and agents have been reported to be associated with AITL, including bacterial infections such as tuberculosis and fungal infections such as cryptococcus (Konig *et al*, 1991;Rho *et al*, 1996). Perhaps most intriguing is the relationship of AITL with a number lymphotropic viruses. The most significant of these is the Epstein–Barr virus (EBV), which is discussed separately below. The presence of other viruses, including human Herpes virus 6 (HHV-6) (Daibata *et al*, 1997;Luppi *et al*, 1993), Herpes virus 8 (HHV-8) (Luppi *et al*, 1996) and hepatitis C virus (Luppi & Torelli, 1996) have been reported in AITL. Other than EBV, the evidence for the role of a viral infection in the pathogenesis of AITL remains tenuous. HHV-6 was reported in seven out of 12 AITL patients identified by PCR (Luppi *et al*, 1993), but immunohistochemistry (Luppi *et al*, 1998) and in situ hybridization studies (Khan *et al*, 1993) showed that the expression of HHV-6 antigens was absent in the T cells and present in reactive plasma cells, which suggests a of a lack of a direct role for HHV-6. Occasional cases of AITL have also been described to have HHV-8 infection by PCR (Luppi *et al*, 1996) but Chadburn and co-workers (Chadburn *et al*, 1997) failed to observe any evidence of HHV8 infection by PCR or immunohistochemistry which, once again, argues against a causal relationship. Although no clear association with human

immunodeficiency virus (HIV) infection has been reported, Muta and Yamano (Muta & Yamano, 2003) report a case of a patient with generalized lymphadenopathy, polyclonal hypergammaglobulinaemia and a lymph node biopsy diagnosis of AITL, whose enzyme immunoassay test for HIV gave a positive (antibody) result for HIV recombinant proteins p24, gp41, and gp36. Western blot analysis provided confirmation of antibodies cross-reacting with HIV p24 gag protein, but HIV RNA was not detected by means of a reverse transcriptase-PCR assay. Thus the patient although not an HIV carrier, gave a "false positive" HIV antibody test, probably due to cross-reacting gammaglobulins. Association with HTLV-1 has been reported but is probably coincidental (Ohshima *et al*, 1998).

1.4.9.3.6 *Clinical features*

AITL typically presents with systemic illness, characterized by B symptoms and generalized lymphadenopathy, often mimicking an infectious process. The majority of patients show hepatosplenomegaly and pruritus, and a skin rash is also seen in a half of patients. The reported frequency of common presenting symptoms and signs observed in AITL are summarized in Table 1.3 (Pautier *et al*, 1999;Siegert *et al*, 1995;Tobinai *et al*, 1988). Laboratory investigations often show the presence of anaemia and occasionally pancytopenia. Typically, there is polyclonal hypergammaglobulinaemia, and both the lactate dehydrogenase and the erythrocyte sedimentation rate (ESR) are often elevated. A significant proportion of patients have circulating autoantibodies, including a positive Coomb's test, cold agglutinins, cryoglobulins and circulating immune complexes. The most common laboratory findings and their frequencies are shown in Table 1.4 (Pautier *et al*, 1999;Siegert *et al*, 1995;Tobinai *et al*, 1988). A number of autoimmune

phenomena have been reported in association with AITL. These include autoimmune haemolytic anaemia (Brearley *et al*, 1979), vasculitis (Hamidou *et al*, 2001;Seehafer *et al*, 1980;Sugaya *et al*, 2001), polyarthritis, rheumatoid arthritis (Pautier *et al*, 1999;Pieters *et al*, 1982), and autoimmune thyroid disease (Ambepitiya, 1989;Pautier *et al*, 1999) which suggests an abnormalitiy in humoral immunity.

Patients also show features of immunodeficiency, believed to be secondary to the T-cell lymphoma, and are thus susceptible to infectious complications. The immune abnormalities and cytokine profiles are discussed below in section 1.9.7.3.4.

Table 1.3. Presenting symptoms and signs in AITL.

Symptoms and signs	Frequency (% of patients)
B symptoms	68-85
Generalised lymphadenopathy	94-97
Splenomegaly	70-73
Hepatomegaly	52-72
Skin rash	48-58
Polyarthritis	18
Ascites / effusions	23-37

Data from grouped from Tobinai *et al* (1988), Siegert *et al* (1992, 1995) and Pautier *et al* (1999)

Table 1.4. Laboratory findings in AITL

Laboratory finding	Frequency (% of patients)
Anaemia	40-57
Other cytopenias	20
Eosinophilia	39
Hypergammaglobulinaemia	50-83
Hypogammaglobulinaemia	9-27
Autoantibodies	66-77
Elevated LDH	70-74
Elevated ESR	45
Bone marrow involvement	61

Data from grouped from Pautier *et al* (1999), Siegert *et al* (1995) and Tobinai *et al* (1988).

1.4.9.3.7 *Pathology*

1.4.9.3.7.1 Histology

The WHO classification (2001) defines of the histology of AITL (see section 1.9.3.2) as a polymorphous infiltrate involving lymph nodes, with a prominent proliferation of HEV and FDCs. The lymph node architecture is at least partially effaced and regressed residual follicles are often present. The polymorphous infiltrate initially involves the paracortex and comprises small, to medium-sized lymphocytes and transformed blasts admixed with eosinophils, plasma cells and histiocytes. Clusters of lymphocytes with pale to clear cytoplasm and distinct cell membranes may be present (Frizzera, 2001;Lee *et al*, 2003b;Lorenzen *et al*, 1994). In the past some authors considered these clusters of clear cells to be essential for the diagnosis of AITL and a useful feature to distinguish lymphoma from "AILD" (Nathwani & Jaffe, 1995). Although earlier studies (Aozasa *et*

al, 1989) attached prognostic value to the presence of clear cells, this has not been validated by more recent studies (Ch'ang *et al*, 1997). In AITL, the lymphocytes may show minimal cytologic atypia. The lymph node sinuses are often preserved but the infiltrate spills over into the perinodal tissue (Frizzera, 2001;Jaffe & Ralfkiaer, 2001a). The presence of eosinophilic amorphous, generally periodic acid-Schiff (PAS) positive, material described in the past in "AILD" (Frizzera *et al*, 1974) and "immunoblastic lymphadenopathy" (Lukes & Tindle, 1975) is not essential for the diagnosis of AITL. Large basophilic blasts (of B-cell phenotype) may be present. Reed-Sternberg-like cells may also be seen (Quintanilla-Martinez *et al*, 1999). On haematoxylin and eosin (H&E) stained sections, the expanded FDC meshwork is seen as ill defined, stretched pale eosinophilic spindle cellular collections (Frizzera, 2001). These are best appreciated and evaluated on immunohistichemistry. Hyperplastic follicles, was for many years believed to exclude the diagnosis of AILD or AITL. However, Ree and colleagues (Ree *et al*, 1998) described 7 cases with molecular genetic evidence of T-cell clonality, that showed hyperplastic follicles, 2 cases of which on subsequent lymph node biopsy showed typical features of AITL. These hyperplastic follicles described were large, with prominent tingible body macrophages and ill-defined borders with indistinct mantle zones. Subsequent to this report, Kojima and co-workers (Kojima *et al*, 2001) described 10 cases of AITL with hyperplastic GCs. In AITL, HEV are prominent and characteristically show arborisation (Frizzera *et al*, 1974;Frizzera, 2001). Lymphocytes may be seen traversing the walls of these vessels. (Frizzera, 2001) In a recent study, Ottaviani and co-workers (Ottaviani *et al*, 2004) have noted that in partial nodal involvement or early AITL, there is minimal displacement of follicles with preservation of GCs and prominent perifollicular sinuses. They also observed that in these nodes the

tumour cells are localized to the paracortex with minimal infiltration into the follicular compartment. They also state that in later stages of AITL, the follicular B-cells shift location to patent trabecular sinuses and stress the importance of shifts in lymphatic patency in the histological features that define AITL.

Although generalized lymphadenopathy is the main presenting sign and the diagnosis of AITL rests on histological examination of the lymph node, many patients have evidence of extranodal involvement at the time of diagnosis. The most frequently involved extranodal sites include the bone marrow, spleen, skin and lungs (Frizzera *et al*, 1974;Ghani & Krause, 1985;Pautier *et al*, 1999;Siegert *et al*, 1992;Siegert *et al*, 1995;Weisenburger *et al*, 1977). As the histologic criteria for diagnosis of AITL have largely been defined for lymph node involvement and relate to the architecture of the lymph node, and since many of the histological features overlap with reactive conditions and other lymphomas, a primary diagnosis of AITL at an extranodal site cannot be made with certainty, without an accompanying lymph node biopsy. Bone marrow is biopsied as a part of staging procedure and is often involved at diagnosis (Pautier, *et al* 1999). There are many reports of skin involvement by AITL (Chang *et al*, 2003;Huang & Chuang, 2004;Martel *et al*, 2000;Matloff & Neiman, 1978;Murakami *et al*, 2001;Suarez-Vilela & Izquierdo-Garcia, 2003), the histology of which is mainly that of a non-specific or atypical T-cell infiltrate. There are a few early reports describing the histology of visceral organ involvement, where the diagnosis, although correlated with lymph node histology, was made largely on a morphological basis with little immunophenotyping and no resort to molecular genetic studies (Moreb *et al*, 1983). Overall, histological appearances in extranodal sites are usually non-specific but mimic some of the features described in the lymph nodes (Brown *et al*, 2001;Ghani & Krause, 1985;Seehafer *et al*,

1980). Cytological features of malignancy can rarely be identified, and tumour involvement can only be reliably shown by immunohistochemistry and molecular clonality analysis (Martel *et al*, 2000;Murakami *et al*, 2001).

1.4.9.3.7.2 Immunophenotype

Immunohistochemistry shows the expansion of the inter-follicular areas by a diffuse infiltrate of CD3 positive T cells (Jaffe & Ralfkiaer, 2001a). In most patients, CD4 positive T cells dominate but there is usually an intermixed population of small CD8 positive T cells (Jaffe & Ralfkiaer, 2001a;Nathwani & Jaffe, 1995). In a few reports (Hodges *et al*, 1997;Watanabe *et al*, 1986) however, CD8 positive cells predominated. By using double layered immunohistochemistry (Feller *et al*, 1988;Namikawa *et al*, 1987), and by PCR analysis of microdissected CD4 and Ki 67 positive cells (Willenbrock *et al*, 2001), it has been shown that the proliferating cells are predominantly of the CD4 positive subtype. In fact even in cases where CD8 positive T-cells predominate, the atypical clear cells and proliferating cells are CD4 positive (Lee *et al*, 2003b). Loss of pan-T-cell markers has been shown by some, (Weiss *et al*, 1986) but not by others (Feller *et al*, 1988;Weiss *et al*, 1986). Lee and co-workers (Lee *et al*, 2003b) reported partial loss of detectable CD4 in the neoplastic cells in some of their cases. A circulating population (believed to be neoplastic) of CD4 positive, CD5 positive, CD7 negative T-cells that express cytoplasmic but not surface CD3, has been reported in AITL (Serke *et al*, 2000). Ree and colleagues (Ree *et al*, 1999) observed BCL-6 expression, in varying intensity (from weak to strong), in small to medium sized CD3 positive T-cells and also in clearly atypical neoplastic cells. They also report that in "AITL with GCs" (3 cases), many GCs were devoid of CD57 positive cells while others

were loosely populated with CD57 positive cells and scattered CD57 positive cells were also seen in the interfollicular areas, suggesting outward migration of intra-follicular T-cells. In their study, double staining for CD57 and BCL-6 showed that 90% of CD57 positive cells were negative for BCL-6, while in the remaining 10% the staining was equivocal, concluding that they are probably separate populations (Ree *et al*, 1999). In a recent study (Rudiger *et al*, 2004), the tumour cell identified by an antibody directed at the Vbeta family used by the tumour clone showed that they were CD3 positive, CD4 positive, CD5 positive, but CD45RO positive, CD45RA negative and CD27 negative, consistent with a terminally differentiated T-cell phenotype.

The B-cell markers CD20 and CD79a highlight the residual follicle centre and mantle zone B cells as well as many of the large transformed blasts and RS-like cells in the interfollicular areas (Lorenzen *et al*, 1994;Smith *et al*, 2000). Transformed blasts including the RS-like cells are highlighted by CD30. CD15 expression has also been reported in the CD30 positive RS-like cells (Quintanilla-Martinez *et al*, 1999). As described by histology, one of the most distinctive features of AITL is the proliferation of FDCs which is best appreciated with immunostaining for the FDC markers CD21, CD23 or CD35 (Bagdi *et al*, 2001;Jones *et al*, 1998;Leung *et al*, 1993;Raymond *et al*, 1997). These expanded FDC meshworks usually surround HEV. One study (Jones *et al*, 1998) describes a zonated, pattern of desmin-positive fibroblastic reticulum cells (FRC) in AITL, but not other PTLs. In this study, the areas of the lymph node with FDCs were reciprocal to the desmin-positive FRC proliferations. As FDC proliferation is an important feature in AITL, to follow (section 1.4.9.3.7.3) is a brief review of the origin, structure and function of this specialized cell.

1.4.9.3.7.3 Follicular dendritic cells (FDCs)

FDCs represent a cell population that provides a meshwork that B-cells lie in to form primary and secondary lymphoid follicles (van Nierop & de Groot, 2002). These cells are derived either from mesenchymal reticular fibroblasts or haematopoietic precursors (Kapasi *et al*, 1994;Kim *et al*, 1994). They possess long cytoplasmic dendrites and are able to retain large immune complexes on their surface for a long period (Wolf-Peeters *et al*, 2001). FDCs present antigens in the form of immune complexes on their surface and switch off the apoptotic machinery of high affinity B-cells, and are thus essential for antigen-triggered B-cell development within the micro-environment of the GC (Lindhout *et al*, 1995;van Eijk & de Groot, 1999;van Eijk *et al*, 2001). FDCs inhibit apoptosis in binding B-cells by up-regulating cFLIP (cellular flice-like inhibitory protein)-expression in attached B-cells, thereby preventing activation of caspase-8 (van Eijk *et al*, 2001). The exact mechanism of this cFLIP up-regulation is not yet known. FDCs also contain high quantities of cystatin A, which acts as an inhibitor of the pro-apoptotic cathepsin recently identified in B-cells (van Nierop & de Groot, 2002). Some studies also suggest a pro-apoptotic role for the FDC in the B-cell selection process, by demonstrating Fas Ligand (FasL) expression in a subset of FDCs in the light zone of the GC, indicating possible Fas-FasL interaction between GC B-cells and FDCs (Hur *et al*, 2000;Verbeke *et al*, 1999). FDCs are also possibly required for the maintenance of memory B-cell clones (Gray *et al*, 1991). All FDCs express the monocyte marker CD14 and the complement receptors CD35 (CR-1), the long isoform of CD21 (CR-2) and CD11b (CR-3) and the Ig Fc receptor CD32 (Dijkstra & Van den Berg, 1991;Tew *et al*, 1990). A subset of FDCs in the light zone of the GC also expresses CD23, a low affinity receptor for IgE and one of the ligands for CD21 (Maeda *et al*, 1992;Payet-Jamroz *et al*,

2001;Wolf-Peeters *et al*, 2001). The CD23 staining coincides with the FasL expressing subset of FDCs (Verbeke *et al*, 1999). FDCs secrete the chemokine B-cell chemoattractant 1 (BCA-1)/CXCL-13, which binds to Burkitt lymphoma receptor 1 (BLR1) /CXCR5 on B-cells, an important step to attract B-cells to the follicle (Estes *et al*, 2004). Adhesion between FDCs and B-cells occurs between ICAM-1/LFA-1 (intercellular cell adhesion molecule-/lymphocyte function-related antigen) and VCAM-1/VLA4 (vascular cell adhesion molecule-1/ very late activation antigen 4) (Freedman *et al*, 1990;Koopman *et al*, 1991). In vitro studies have shown that FDCs adhere to malignant B-cells by the adhesion molecules VLA4 and VCAM-1 (Freedman *et al*, 1992).

Unlike other antigen presenting cells, the FDCs do not internalize and process antigens, and do not synthesize MHC-II molecules (Denzer *et al*, 2000). They however carry peptide-MHC class II expressing microvesicles (exosomes derived from B-cells) on their surface, enabling interaction with CD4 positive T-cells (Denzer *et al*, 2000). Interaction with T-cells also occurs by CD40-CD40 ligand binding (Banchereau, *et al* 1994). FDCs up-regulate chemokine receptor CXCR4 on CD4 positive cells, but inhibit signaling through this receptor by up-regulating regulator of G protein signaling (RGS), RGS13 and RGS16 (Estes *et al*, 2004). Therefore, despite high levels of CXCR4, GC T-cells do not migrate to the CXCR4 ligand, CXCL12 (Estes *et al*, 2004). Nevertheless as GC CD4 positive T-cells migrate to BCA-1/CXCL13 produced by FDCs, the latter appears to play an active role in GC T-cell migration (Estes *et al*, 2004). In vitro studies have also shown that highly purified FDCs are able to induce the proliferation of allogeneic T-cells or T-cell lines (Butch *et al*, 1994).

In a recent study it was shown that FDCs and endothelial cells in AITL express FasL and the tumour T-cells express Fas (CD95) and caspase 3, indicating Fas-FasL interaction between the neoplastic T-cell and the FDC (Kim, *et al* 2002) (to be discussed below). However, despite these immunohistochemical findings, as of now, there is however no direct evidence demonstrating interactions between FDCs and neoplastic T-cells in AITL.

1.4.9.3.8 *Immunology /cytokines in AITL*

There appears to be a substantial immune activation in the peripheral blood and lymph nodes of AITL patients compared with the reactive lymph nodes or other PTLs. The evidence for this includes increased levels of serum soluble interleukin 2 (IL-2) receptor, CD30 and CD8 molecules (Pizzolo *et al*, 1990), and also the expression of an array of cytokines such as tumour necrosis factor alpha, lymphotoxin, IL-1 beta, IL-2, IL-4, IL-6, IL-13 and interferon gamma (Foss *et al*, 1995;Ohshima *et al*, 2000). Recent studies examining the expression of T-cell activation markers have shown an increased expression of the TNF receptor family member CD134 (OX40) (Jones *et al*, 1999;Tsuchiya *et al*, 2004), chemokine receptor CXCR3, (Ishida *et al*, 2004;Jones *et al*, 1999;Ohshima *et al*, 2004;Tsuchiya *et al*, 2004) CD69, which is a marker of early T-cell activation (Dorfman & Shahsafaei, 2002) and T-box transcription factor, T-bet (Dorfman *et al*, 2003). These molecules are preferentially associated with the Th-1 phenotype in normal T-cells, suggesting that Th-1-type differentiation is a feature of the neoplastic cell in AITL. AITL express T-cell factor-1 (TCF-1) and lymphoid enhancer factor-1, components of the WNT/β-catenin signal transduction pathway, a feature also

seen in other "Th-1-like" PTL, but not "Th-2-like" PTL such as ALCL (Dorfman *et al*, 2003).

In contrast to this marked immune activation, the functional studies performed on T cells recovered form lymph nodes and peripheral blood of AITL patients have shown defective T-cell responses supporting an underlying immunodeficiency. The immune abnormalities reported include a decrease in circulating T-cells, an altered CD4: CD8 ratio, altered absolute levels of CD4 negative or CD8 positive T-cells, and high percentages of activated T cells (CD8+/HLA-DR+), defective T-cell response in vitro to the phytohaemagglutinin (PHA) mitogen, and minimal helper and enhanced in vitro suppressor functions (Ganesan *et al*, 1987;Pizzolo *et al*, 1983). Lymphopaenia has been reported with a frequency of ~50% and skin test anergy in 68% of patients (Archimbaud *et al*, 1987).

Vascular endothelial growth factor (VEGF), one of the main angiogenic cytokines in human solid tumours, is expressed at high levels by the stromal cells in AITL (Foss *et al*, 1997). Over-expression of VEGF-A gene and expression of VEGF-A protein have been demonstrated in both microdissected lymphoma cells and endothelial cells, while VEGF-R co-expression has been shown in endothelial cells. Zhao and co-workers (Zhao *et al*, 2004) also report a correlation between increased levels of VEGF-A gene expression and survival. It has been hypothesized that VEGF expression may be responsible for the vascular proliferation observed in AITL. Interestingly, the increased VEGF signal appears to correlate with the number of mast cells infiltrating the tumour, suggesting that mast cell activation may also play a role in the pathogenesis (Fukushima *et al*, 2001).

1.4.9.3.9 *Possible normal counterpart of AITL:*

Mature CD4 positive T-cell is considered the putative "cell of origin" (WHO, 2001). The findings of Rudiger and colleagues (Rudiger *et al*, 2004) that the tumour cells were of the CD45RO positive, CD45RA negative, CD27 negative phenotype (see above in section 1.5.3.7) suggest that the neoplastic T-cell in AITL is derived from a terminally differentiated CD4 helper T-cell. Cytokine profiling suggests a Th1 cell-type (Dorfman & Shahsafaei, 2002;Dorfman *et al*, 2003;Ishida *et al*, 2004;Jones *et al*, 1999;Ohshima *et al*, 2004;Rudiger *et al*, 2004;Tsuchiya *et al*, 2004), and the close association with the follicle center/FDC microenvironment suggests that a CD4 positive GC T-cell origin is a possibility.

1.4.9.3.10 *EBV infection*

Prior to EBV infection in AITL, it may be appropriate to briefly review EBV infection in healthy individuals, in the immune-deficient and its association with lymphomas.

1.4.9.3.10.1 EBV primary infection and latency in healthy individuals

Over 95% of the world's population is infected with EBV, a gamma herpes virus with a double stranded DNA genome of 172 kb pairs. EBV infects mainly B-cells, but they are also known to infect T-cells and epithelial cells (Kuppers, 2003). EBV infection usually occurs in childhood and results in an asymptomatic or mild self-limiting illness. If the infection is delayed until adolescence or young adulthood, infectious mononucleosis occurs in around half the infected individuals (Kuppers, 2003). In healthy carriers, primary infection occurs via the oropharyngeal route with productive infection of B-cells or epithelial cells. The released virus then infects B-cells circulating through the

oropharynx, resulting in a latency type 3 infection with expression of nine viral proteins, the nuclear antigens Epstein Barr virus nuclear antigen (EBNA) 1, 2, 3A, 3B, 3C, the latent membrane proteins (LMPs) 1, 2A and 2B, 2 small RNAs and transcripts from the *Bam*H1 A region of the genome (Tierney *et al*, 1994). This type of latency is highly immunogenic and induces a massive, virus-specific and non-specific T-cell response. A small number of B-cells, escape this immune response by expressing a minimal form of latency in which only one viral protein (LMP-2) is expressed (Tierney *et al*, 1994). The virus persists in this latent state for the life of the individual (Yao *et al*, 1985).

1.4.9.3.10.2 EBV in the immunosuppressed

In those with impaired cellular immunity increased virus reactivation occurs (Babcock *et al*, 2000) and in immunosuppressed individuals an uncontrolled EBV-driven B-cell proliferation may lead to a lymphoproliferative disorder (Ho *et al*, 1988;Shapiro *et al*, 1988).

1.4.9.3.10.3 EBV-infection and lymphomas

The EBV positive B-cell lymphomas include CHL, Burkitt lymphoma, which occur in the immunocompetent and also as mentioned above, in the immunocompromised (Brousset *et al*, 1991;Carbone, 2003;Klumb *et al*, 2004;Niedobitek *et al*, 1995;Tinguely *et al*, 1998). Pyothorax associated lymphoma is associated with EBV in seemingly immunocompetent individuals (Fukayama *et al*, 1993;Kanno & Aozasa, 1998;Sasajima *et al*, 1993). Lymphomatoid granulomatosis, an angiocentric and angiodestructive lymphoproliferative disease comprising EBV positive B-cells amidst numerous reactive T-cells, often occurs in the setting of immunodeficiency (Guinee, Jr. *et al*, 1994;Haque

et al, 1998;Jaffe & Wilson, 2001;Nicholson *et al*, 1996;Taniere *et al*, 1998), as does the Human Herpes virus-8 (HHV-8) associated primary effusion lymphoma which usually shows co-infection with EBV and develops most often in the setting of HIV infection (Banks & Warnke, 2001;Horenstein *et al*, 1997;Nador *et al*, 1996).

Of the mature T/NK-cell neoplasms, EBV is universally associated with extranodal NK/T-cell lymphoma nasal-type and in the majority of aggressive NK-cell leukaemias (Chan *et al*, 2001a;Chan *et al*, 2001b;Kanavaros *et al*, 1993;Nava & Jaffe, 2005). EBV- has been reported in bystander B-cells in cases of PTLu and have been associated with a poor outcome (d'Amore *et al*, 1996).

1.4.9.3.10.4 EBV and AITL

A characteristic feature of AITL, seen in over 95% of all patients, is the presence of increased numbers of EBV-infected cells compared with both normal lymph nodes and PTLs (Weiss *et al*, 1992). Initial studies using Epstein Barr virus encoded RNA (EBER)- in situ hybridization and comparing the results with immunohistochemical sections stained for T- and B-cell markers suggested the EBV-infected cells might be within both the T-cell and B-cell population (Anagnostopoulos *et al*, 1992). However, studies using double immunohistochemistry, in situ hybridization and microdissection have shown that virtually all cells infected by EBV are B cells, and that EBV infection is unlikely to play a primary role in the lymphomagenesis of AITL (Brauninger *et al*, 2001;Ohshima *et al*, 1994;Weiss *et al*, 1992). In AITL, the EBV infected B-cells are somatically mutated consistent with a GC or memory gentotype. The majority of EBV infected B-cells show ongoing hypermutation during clonal expansion (Brauninger *et al*, 2001). It has also been shown that many of these clones have destructive "crippling" mutations

without selection for expression of a functional antigen receptor and that the somatic hypermutation process is active even in these clones (Brauninger, *et al* 2001). These findings suggest that EBV infection may in some way influence the survival of these cells that would otherwise be destined to undergo apoptosis. However, in AITL, occasionally, EBV negative "crippled" B-cells can also survive and expand (Brauninger *et al*, 2001). The EBV protein expression pattern in AITL B cells is usually consistent with latency (Anagnostopoulos *et al*, 1995;Zettl *et al*, 2002). The causes for the expansion of EBV-infected cells are not known. It is likely that an underlying immunodeficiency with reduced cytotoxic activity, and the presence of growth factors favouring the outgrowth of EBV-infected cells play roles. The EBV-infected cells have a variable cytology, with some having an immunoblast-like and others having an RS-like appearance. They account for most of the large B cells present in the interfollicular zone of the AITL lymph nodes. EBV-associated large B-cell lymphoma (Abruzzo *et al*, 1993;Matsue *et al*, 1998;Xu *et al*, 2002a;Zettl *et al*, 2002) and EBV positive CHL (Nakamura *et al*, 1995) have been reported as complications of AITL. Knecht and colleagues (Knecht *et al*, 1995) reported the presence of an LMP-1 deletion mutant, identical to that reported in CHL.

1.4.9.3.11 *Clonality analysis*

Before the availability of molecular tools that can demonstrate the presence of expanded clones of T and B cells, the neoplastic nature of AITL was not fully appreciated and the disease was considered to be a premalignant state from which high-grade, large cell lymphomas occasionally arose. When Southern blotting technology became available, Weiss and colleagues were the first to investigate the clonality of lymphocytes in AITL

(Weiss *et al*, 1986). They demonstrated the presence of clonal T-cell populations, not only in patients with histologically apparent lymphoma, but also in patients diagnosed as AILD who lacked the cytological features of malignancy. This was followed by a number of similar publications confirming the presence of a monoclonal T-cell population in virtually all patients with AILD /AITL (Feller *et al*, 1988;Lipford *et al*, 1987;O'Connor *et al*, 1986;Ree *et al*, 1998;Smith *et al*, 2000;Tobinai *et al*, 1988;Willenbrock *et al*, 2001). Hodges and colleagues (Hodges *et al*, 1997) report a case showing an oligoclonal T-cell proliferation on PCR and sequencing where most of the infiltrating atypical T-cells (CD3 and CD8 positive) belonged to the Vβ5.1 family. In the study by Willenbrock and co-workers (Willenbrock *et al*, 2001), they report the over-usage of the V-gene segment, Vβ13S1, while Smith and co-workers (Smith *et al*, 2000) found restricted usage of Vβ2S1 in 4/11 cases. Restricted usage of certain V-gene segments in AITL raises the possibility of specificity of the neoplastic cell for a yet unknown, shared common antigen, in some of the cases. However, in their series, Willenbrock and co-workers (Willenbrock *et al*, 2001), failed to demonstrate an MHC II allele that was common to all cases with Vβ13S1-expressing neoplastic clones. The results of clonality analyses from some reported series are summarized in Table 1.5. One of the unusual observations to be made during these studies was the presence of an expanded monoclonal B-cell population in a significant minority of patients. This has led to the suggestion that AITL is a mixture of T-cell lymphomas and B-cell lymphomas, but not a single clinicopathological entity. However, the current evidence suggests that these patients also fall within the framework of AITL. These patients typically exhibit increased numbers of EBV-infected B-large cells, and it is thought that the B-cell clone detected by molecular analysis of whole lymph node extracts lie within

this population. It is likely that this is an EBV-driven lymphoproliferation that occurs secondary to the immuno-deficiency associated with the underlying AITL, perhaps similarly to other EBV-driven lymphoproliferations that are associated with immunosuppression (Zettl *et al*, 2002). Not surprisingly, a subset of AITL patients go on to develop full-scale EBV-associated, diffuse, large B-cell lymphomas (Abruzzo *et al*, 1993;Knecht *et al*, 1995;Zettl *et al*, 2002) or Burkitt's lymphoma. (Mazur *et al*, 1979).

Table 1.5. T- and B-cell clonality in AITL.

Study	Method	T-cell clonal Patients	B-cell clonal Patients
Weiss *et al* (1986)	SB	8/10	0/10
Lipford *et al* (1987)	SB	5/5	4/5
Feller *et al*, (1988)	SB	24/24	7/24
Tobinai *et al* (1988)	SB	8/10	0/10
Lorenzen *et al* (1994)	PCR	9/15	5/15
Ree *et al* (1998)	SB/PCR	7/7	0/7
Smith *et al* (2000)	PCR	21/22	9/22
Willenbrock *et al* (2001)	PCR	12/18	1/13

SB, Southern blotting; PCR, polymerase chain reaction.

1.4.9.3.12 *Genetic changes*

The studies on the genetic changes occurring in AITL have been hampered by a number of factors, including the relative rarity of the tumour and dilution of tumour cells by a large number of reactive cells. Most of our knowledge on genetic changes comes from cytogenetic-based studies. These are summarized in Table 1.6. Approximately 90% of AITL patients have cytogenetic alterations observed by the use of combined metaphase and interphase cytogenetics (Cosimi *et al*, 1990;Godde-Salz *et al*, 1987;Kaneko *et al*, 1982;Lepretre *et al*, 2000;Schlegelberger *et al*, 1990a;Schlegelberger *et al*, 1994b). Clonal chromosomal aberrations are seen in approximately 70% of the patients. Trisomy

3, trisomy 5 and gain of an X chromosome are the most frequent recurrent abnormalities seen in AITL, but these are also present in other PTLs. These abnormalities have also been reported using fluorosence in situ hybridization (FISH) (Kumaravel *et al*, 1997). Trisomy 3 and 5 are reported to be less frequent in AITL in the Far East than in the West (Chen *et al*, 2004). Schlegelberger and colleagues showed that half the patients harboured cytogenetically unrelated clones, which is a unique phenomenon that is exceptional in other lymphomas (Schlegelberger *et al*, 1990a;Schlegelberger *et al*, 1994b). This was hypothesized to be consistent with the stepwise development of chromosomal aberrations in AITL. The steps being the appearance of chromosomal abnormalities in different cells because of genetic instability and the impaired elimination of aberrant cells due to the immune defect, followed by the establishment of chromosomally aberrant clones, and finally a cytogenetically detectable level of monoclonal proliferation. However, at the first step, T-cell receptor gene rearrangement shows clonal T-cell proliferation, indicating that the abnormal tumour clone is present at the onset, although perhaps not detectable by cytogenetic methods that have a low sensitivity. The presence of T-cell clones and the EBV-driven expansion of B-cell clones raises the question as to which cells harbour the aberrant karyotype. In one patient, using a method involving the simultaneous demonstration of immunophenotype and karyotype, it was shown that the aberrant mitoses with trisomy 3 were CD3 positive and, therefore, T cells (Schlegelberger *et al*, 1990b). It is possible that the non-clonal aberrations seen in AITL originate from EBV-infected B cells that are likely to have an in-vitro growth advantage. The lower proportion of aberrant cells reported on interphase cytogenetics compared with metaphase cytogenetics is consistent with the morphology of AITL, with a few neoplastic cells amidst an abundance of reactive cells

(Schlegelberger *et al*, 1994a). In a study assessing the significance of cytogenetics on clinical outcome, only the presence of complex aberrant clones was determined to be an independent prognostic factor, and trisomy 3 had no effect on survival (Schlegelberger *et al*, 1996). Genes known to be critical in lymphomagenesis have rarely been studied in AITL. Petit and colleagues examined p53 expression and mutations, and report that both are rare, suggesting that this pathway is not altered in the majority of AITL patients (Petit *et al*, 2001). The other gene that has been examined is BCL-6. Rearrangements and /or mutations of the 5 non-coding region of the BCL-6 gene play a role in the development of diffuse large B-cell lymphoma (DLBCL). Despite the reports of BCL-6 expression in the tumour cells in AITL (Ree *et al*, 1999), no mutations were detected in the 5 non-coding region of the gene of 33 cases of PTLs which included 2 cases of "angioimmunoblastic lymphadenopathy" and 2 cases of "immunoblastic lymphoma" (Kerl *et al*, 2001).

Table 1.6. Cytogenetic alterations in AITL

Study	Patients with cytogenetic alterations	Patients with clonal cytogenetic alterations	Cytogenetic alterations frequently encountered
Kaneko *et al* (1982)	5/6	4/6	+3in 4/6 patients +5, +15, +19, +21, +22 in 2/6 patients
Godde-Salz *et al* (1987)	13/18	7/18	+3, +5
Kaneko *et al* (1988)	10/10	9/10	+3, +5, 6q-
Cosimi *et al* (1990)	3/6	-	t(7;14)(q35;q11)
Schlegelberger *et al* (1990a)	35/42	25/42	+3, +5, +X
Schlegelberger *et al* (1994a)	32/36	32/36	+3, +5, +X

All studies have used metaphase cytogenetics; Schlegelberger *et al* (1994) have used both metaphase and interphase cytogenetics. +3, +5,+15, +19, +21, +22 and +X refer to trisomy of chromosomes 3, 5, 15, 19, 21, 22 and X respectively.

1.4.9.3.13 *Diagnosis*

The clinical syndrome of AITL overlaps with a wide range of inflammatory and neoplastic processes, and the changes in peripheral blood and bone marrow are usually non-specific. Fine needle aspiration of the enlarged lymph node may be helpful (Dey *et al*, 1996;Kerl *et al*, 2001;Ng *et al*, 2002;Yao *et al*, 2001), but is rarely diagnostic because cytological appearances can be within normal limits and architectural features cannot be obtained. For the same reasons, a needle core also has limited value. The diagnosis of AITL can only be achieved by biopsy and histological examination of the enlarged lymph nodes, where characteristic morphological features can be best appreciated.

1.4.9.3.14 *Clinical outcome*

Publications regarding the outcome and clinical management of AITL are limited due to the rarity of the disease. Most of the information is based on retrospective studies, small patient numbers and a limited number of case reports. The clinical outcome of the AITL remains dismal, with a median survival of less than 36 months and a 5-year survival of around 30–35% (Pautier *et al*, 1999;Pellatt *et al*, 2002;Siegert *et al*, 1992;Siegert *et al*, 1995). Most patients die of infectious complications rather than tumour load, suggesting that an underlying immunodeficiency significantly contributes to the AITL-associated mortality. In the few patients where there has been a record of consecutive lymph node biopsies, these have included, subtle changes with follicular hyperplasia in the first biopsy and "typical" AITL in the second biopsy (Ree *et al*, 1998). There are some earlier reports of histologic transformation to an "immunoblastic lymphoma" (Bauer, *et al*

1982; Pangalis, *et al* 1983), but due to the limited immunophenotyping available at the time it is difficult to determine whether this transformation is of T- or B-cell phenotype. As mentioned previously in the section on EBV and AITL (section 1.5.3.5), there are reports of EBV-positive DLBCLs complicating AITL (Abruzzo *et al*, 1993;Matsue *et al*, 1998) and of EBV-positive CHL (with no histologic, immunophenotypic or genetic evidence of T-cell lymphoma) subsequent to AITL (D'Arrigo *et al*, 1985;Melato *et al*, 1983;Nakamura *et al*, 1995). Higuchi and co-workers (Higuchi *et al*, 1998) describe a case of "immunoblastic lymphadnopathy-like T-cell lymphoma" which on biopsy during chemotherapy, was found to be overrun by a massive plasma cell proliferation giving rise to a paraproteinaemia with biclonal peaks.

Both single agent and combination chemotherapeutic regimens such as CHOP (cyclophosphamide, doxorubicin, Oncovin, prednisone), CVP (cyclophosphamide, vincristine, prednisone), VAP (vincristine, asparaginase, prednisone), steroids with or without cyclophosphamide, and high-dose methylprednisolone, prednisone with or without COPBLAM (cyclophosphamide, Oncovin, prednisone, bleomycin, Adriamycin, Matulane) or IMVP-16 (ifosfamide, methotrexate, VP-16) have been reported (Awidi *et al*, 1983;Colbert *et al*, 1982;Pautier *et al*, 1999;Siegert *et al*, 1992;Siegert *et al*, 1995). Although a complete remission rate of 50% can be achieved with combination chemotherapy, relapse rates remain high. Overall, combination chemotherapy appears to be superior to steroids alone (Pautier *et al*, 1999;Siegert *et al*, 1992). Other therapeutic approaches, including low-dose methotrexate together with steroids (Gerlando *et al*, 2000), fludarabine (Hast *et al*, 1999;Ong *et al*, 1996;Tsatalas *et al*, 2003) and 2-chlorodeoxyadenosine (Sallah & Bernard, 1996;Sallah *et al*, 1999) can be beneficial, but again studies are based on a small number of patients, which does not allow

statistically significant conclusions. Interferon-alpha has been used for consolidation–maintenance therapy following conventional treatment to prolong chemotherapy-induced remissions as a result of its differentiating, immunomodulating and anti-proliferative effects (Feremans & Khodadadi, 1987;Hast & Gustafsson, 1991;Pautier *et al*, 1999;Schwarzmeier *et al*, 1991;Siegert *et al*, 1991). In the majority of the patients, the remission duration is variable but is not longer than that observed with conventional treatments. Cyclosporin A, which has a suppressive effect on the immune system, most notably on T cells, and a direct cytotoxic/apoptosis-inducing effect on lymphocytes, has also been given (Advani *et al*, 1997;Murayama *et al*, 1992;Takemori *et al*, 1999). Its combined effects on neoplastic T cells may play an important role in the achievement of remission, but once again at present, studies are limited to a few case reports. There is however, a phase II clinical trial, set up to evaluate the use of cyclosporine A in the treatment of recurrent or relapsed AITL (Eastern Coorporative Oncology Group, 2003), the results of which will determine its application on a wider basis. Thalidomide has been used as anti-angiogenetic agent in a few patients, either following relapse or in refractory AITL, with promising results (Strupp *et al*, 2002). There are reports that patients treated after relapse with high-dose chemotherapy followed by autologous bone marrow/blood stem cell transplantation responded favourably (Blystad *et al*, 2001;Lindahl *et al*, 2001;Pautier *et al*, 1999;Schetelig *et al*, 2003;Schmitz *et al*, 1991) and a few recent publications on its effectiveness as the first line of treatment, in selected patients (Reimer *et al*, 2004;Schetelig *et al*, 2003). One of the latter studies (Reimer *et al*, 2004) is a prospective study of 30 patients that includes 12 patients with AITL but needs longer follow up to draw definite conclusions on survival benefits.

EBV-driven DLBCL is a complication of AITL that may benefit from anti-viral therapy (Battegay *et al*, 2004). Park, and co-workers (Park *et al*, 2002) report a case of EBV-related DLBCL following autologous transplantation for AITL, that was successfully treated by Rituximab (monoclonal anti-CD20 antibody). The success of Rituximab plus CHOP over CHOP alone for the treatment of DLBCL in the elderly has already been evaluated in a randomized clinical trial by Groupe d'Etudes des Lymphomes de l'Adulte (GELA), France (Coiffier, 2002). Rituximab in conjunction with CHOP has also shown good results in 4 patients with AITL rich in large B-cells (Joly *et al*, 2004) and requires evaluation in a phase II trial before application on a wider basis.

1.4.9.3.15 *Problems related to definition and diagnosis*

Unlike in the classification of B-cell lymphomas where many of the neoplasms have well defined morphologic and immunophenotypic features, the world Health Organization (WHO) classification emphasizes that due to the lack of specific morphologic, immunophenotypic features, and in most instances, the lack of specific genotypic features, clinical features play an important role in the subclassification of mature T/NK-cell neoplasms.

In AITL when the typical morphology with pronounced FDC proliferation is encountered, diagnosis is fairly straightforward. Although reproducibility by experts is reported to be high (Rudiger *et al*, 2002), diagnosis can be complicated by the fact that the neoplastic T-cells often are in the minority, and are greatly outnumbered by the reactive cells (Willenbrock *et al*, 2005). Cytologic features of malignancy may not be evident in the tumour cells and many of the morphologic features considered "characteristic" of AITL, are not specific and overlap with reactive conditions and other

lymphomas. The presence of a "polymorphous infiltrate" and "prominence of HEV" shows overlap with the histology of reactive hyperplasia and PTLu. Even the "clusters of clear cells" when present are not specific, as it may be a seen in PTLu. The "expanded FDC meshwork that usually surrounds HEV" is the only relatively specific feature that is useful in distinguishing AITL from other PTL. Although this feature is well developed in "typical" AITL, it may be fairly subtle in "early" cases with hyperplastic follicles. In this situation, the distinction from reactive lymphoid hyperplasia on morphology alone and from "T-zone morphologic variant" of PTLu, even with the support of immunophenotyping and molecular genetic analysis, is difficult and at times impossible. Those with a high content of epithelioid histiocytes show a histiologic overlap with Lennert-morphological variant of PTLu (Patsouris *et al*, 1989).

The prominence of regressed follicles in some cases may cause confusion with Castleman disease (Frizzera, 2001). There is an older report of "AILD" which followed cutaneous kaposi's sarcoma (Suster *et al*, 1987) and another two reports of the two conditions occurring simultaneously in the same patient (Friedman-Birnbaum *et al*, 1985;Kluin-Nelemans *et al*, 1984). Considering the morphologic overlap between the two conditions, these may represent examples of HHV-8 related Castleman disease rather than AITL.

The presence of prominent EBV-infected B-blasts may lead to an erroneous diagnosis of a DLBCL, especially if there is outgrowth of a dominant clone giving a monoclonal molecular genetic result. Reed Sternberg-like cells in a mixed "inflammatory" background mimics classical Hodgkin Lymphoma, mixed cellularity subtype.

1.5 Aims of this project

Despite the advances made in the last 3 decades, the biology of the disease remains poorly understood, and there is no specific phenotypic marker that distinguishes the early cases of AITL and those with less typical histology, from reactive conditions and other lymphomas. Except for a few anecdotal reports there is no proper study into the histology of disease progression. No aetiological agent has yet been identified and there is no consensus regarding the approach to management.

The general aim of this study is to further characterize AITL.

The specific aims are:

1. Identify a specific phenotypic marker for diagnosis of AITL that would help distinguish the disease from reactive proliferations and other nodal PTLs.
2. Study the histologic progression of the disease in sequential biopsies.
3. Study the cell of origin
4. Study the role of EBV and HHV8 infection in the development and progression of AITL.

Chapter 2

MATERIALS AND METHODS

2.1 Detection kits used for immunohistochemistry

ChemMate™ Detection Kit, Peroxidase/DAB, Rabbit/Mouse [*DakoCytomation, Cambridge, UK (Code no: 5001)*] containing ChemMate™ Link, Biotinylated Secondary Antibodies (AB2), ChemMate™ Streptavidin Peroxidase (HRP), ChemMate™ DAB+ Chromogen,and ChemMate™ HRP Substrate Buffer were used for single layered immunohistochemistry and for detection of the 1st antibody during double-layered immunohistochemistry.

ChemMate™ Detection Kit, APAAP, mouse [*DakoCytomation, (Code no: 5000)]*, was used for visualization of the 2nd antibody in double layered immunohistochemistry. The reagents used from this kit were the ChemMate™ Link, Secondary Antibody (LINK) and ChemMate™ APAAP Immunocomplex (APAAP). The chromogen used for this step was fast blue, as the blue would contrast well with the brown DAB stain. For this purpose, Vector® Blue Alkaline Phosphatase Substrate Kit III [*Vector Laboratories, Peterborough, UK (Cat. No. SK-5300)*] was used.

2.2 Solutions

2.2.1 Solutions used in immunohistochemistry and in-situ hybridization

2.2.1.1 Tris buffered saline pH 7.6 (TBS)

6.05 g of Tris[hydroxymethyl] aminomethane (*Sigma Chemical Co Ltd, Poole, Dorset, UK*) and 80 g NaCl (*BDH Laboratory Supplies, Merck Ltd, Lutterworth, Leicestershire, UK*) were dissolved in 8 litres of distilled water, pH was adjusted to 7.6 with 1 M HCl (*BDH*) and the volume was brought to 10 litres with distilled water.

2.2.1.2 TBS-Tween

Tween 20 (*Sigma*) was added to TBS to give a final concentration of 0.05%.

2.2.1.3 Tris-HCl buffer (pH 8.2)

Stock A (0.2M Tris) – was made up by dissolving 2.42 g of Tris[hydroxymethyl] aminomethane (*Sigma*) in 100 ml of distilled water.

Stock B (0.2M HCl) was made up by mixing 1.7 ml of 1M HCl (*BDS*) in 100 ml of distilled water. To obtain pH 8.2, 25 ml of Stock A and 11 ml of Stock B were mixed together.

2.2.1.4 0.1M Tris-HCl (pH 9.5)

0.1M Tris-HCl, pH 9.5 buffer consisted of 10 ml of 0.1 M Tris solution, made up by dissolving 1.21 g of Tris[hydroxymethyl] aminomethane (*Sigma*) in 100 ml of distilled water. The pH was adjusted as required with 1M HCl.

The solution was filtered through Whatman No.1 filter paper (*BDH*) and stored at 4°C.

2.2.1.5 1M Tris-HCL (pH 7.5)

12.0 g of tris was dissolved in 40 ml of distilled water, brought to 100 ml final volume with distilled water and the pH tritrated to 7.5 with 1M HCl . The solution was filtered through Whatman No.1 filter paper (*BDH*) and stored at 4°C.

2.2.1.6 Peroxidase block solution

This solution was always prepared fresh just prior to use and composed of 200 µl of H_2O_2 (hydrogen peroxide) (*Sigma*) in 12 ml of methanol (*BDH*).

2.2.1.7 ChemMate™ HRP Substrate Buffer

Commercially available (*DakoCytomation*,), ready to use buffered solution containing hydrogen peroxide and preservative.

2.2.1.8 Solution of Chemmate™ DAB+ Chromogen in Chemmate™ HRP substrate buffer

1 volume of ChemMate™ DAB+ Chromogen (*DakoCytomation*) that contains 50 × concentrated 3,3'-diaminobenzidine tetrahydrochloride in organic solvent, dissolved in 50 volumes of ChemMate™ HRP Substrate Buffer (*DakoCytomation*), made up just prior to use.

2.2.1.9 Alkaline Phosphatase-Substrate solution

Commercially available, Vector® Blue Alkaline Phosphatase Substrate Kit III [*Vector Laboratories (Cat. No. SK-5300)*] was used. The Vector® Blue substrate working solution was made up immediately before use in a test tube. The kit provides all reagents except the buffer. Two drops of Reagent 1 were added to 5ml of Tris-HCl, pH 8.2 buffer

and mixed well. This was followed by 2 drops of Reagent 2 followed by mixing, and finally 2 drops of Reagent 3 followed by mixing.

2.2.1.10 Citrate buffer pH 6.0

Monohydrate citric acid (*BDH*) of 2.1 g was dissolved in 1 litre of distilled water and pH was adjusted to 6.0 with 1 M HCl.

2.2.1.11 Dako target retrieval solution pH 6.0

60 ml of Dako target retrieval solution pH 6.0 (*DakoCytomation*) was mixed with 540 ml of distilled water.

2.2.1.12 Dako target retrieval solution pH 9.9

60 ml of Dako target retrieval solution pH 9.9 (*DakoCytomation*) was mixed with 540 ml of distilled water.

2.2.1.13 2 × SSC solution

The solution was made up by adding 5 ml of 20 x SSC (3.0 M Sodium Chloride, 0.3 M Sodium Citrate, pH 7.0) (*Sigma*) to 45 ml of double distilled water (DDW).

2.2.1.14 1 × SSC solution

The solution was made up by adding 2.5 ml 20 × SSC (*Sigma*) to 47.5ml of DDW.

2.2.1.15 Proteinase K buffer (for in situ hybridisaton)

2ml of 0.5M EDTA (ethylene diamine tetra-acetic acid) (*Sigma*) was added to 10 ml of 1M Tris-HCl (pH 7.5) and the volume brought up to 200 ml with DDW. The final concentration of Tris-HCl was 50 mM and EDTA 5mM.

2.2.1.16 Hybridisation buffer

1. 250 µl Sodium dodecyl sulphate (SDS) (*BDH*) was dissolved in 10 ml of DDW (heated to 60°C using a hot water beaker on a hot plate).
2. In a separate bottle (bottle 1), 1g dextran sulphate (*Sigma*) was added to 1 ml of 20 × SSC (Sigma) and dissolved at 60°C.
3. In another bottle (bottle 2), 25 mg Ficoll (*Sigma*), 25 mg Polyvinyl_Pyrrolidone (PVP) (*Sigma*), 50 mg sodium pyrophosphate (*Sigma*) were added to 1 ml of DDW and dissolved with heat as before. 25 mg bovine serum albumin (BSA) (*Sigma*), was then added with 2.5 ml of 2M Tris (*Sigma*) into the second bottle.
4. Contents of the two bottles (bottles 1 and 2) were then mixed together and 5 ml of Formamide (*Sigma*) and 250 µl of SDS (from step 1) added, and the contents gently mixed. 1ml aliquots were then prepared and the hybridisation buffer stored at -20°C.

2.2.1.17 Nitroblue tetrazolium chloride (NBT) – 5-Bromo-4-Chloro-3-Indolyl phosphate (BCIP) substrate solution

Substrate kit was purchased ready-made from *Life science technologies, Paisley, UK.* The substrate solution was prepared just prior to use by mixing 44 µl of NBT solution and 33 µl of BCIP solution to 10.0 ml of 0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl, 50 mM $MgCl_2$ (*Sigma*).

2.2.1.18 0.1% Nuclear Fast Red in 5% Aluminium Sulphate solution

5g aluminium sulphate (*Sigma*) was dissolved in 100 ml of distilled water, heated and stirred to dissolve 0.1g nuclear fast red (*Sigma*) to achieve a final concentration of 0.1% nuclear fast red. The solution was then filtered and stored at 40°C and re-filtered prior to use.

2.2.2 Solutions used in PCR, cloning and sequencing

2.2.2.1 DNA extraction

2.2.2.1.1 *Proteinase K/ (extraction) digestion buffer*

Concentration used was 300mg/ml proteinase K (*Sigma*) in 1× PCR buffer. To make up 1 ml of extraction buffer, 100 µl of 10 × PCR buffer (*Promega, Southampton, UK*) and 30 ml proteinase K (10mg/ml stock – *Sigma*) were added to 870 µl of ultrapure water (*Sigma*).

2.2.2.2 PCR

2.2.2.2.1 *10×PCR buffer*

Commercially available 10x PCR buffer (*Promega*) containing 100mM Tris pH 9, 500mM KCl and 1% Triton X-100.

2.2.2.3 Electrophoresis

2.2.2.3.1 *10% Ammonium persulphate solution*

1 g of ammonium persulphate (*Bio-Rad, Hertfordshire, UK*) was dissolved in 10 ml of distilled water.

2.2.2.3.2 *10 × Tris Borate EDTA (TBE)*

108g Tris, 55g boric acid (*Sigma*) and 9.3g EDTA (*Sigma*) are dissolved in distilled water to a volume of 1 litre.

2.2.2.3.3 *Electrophoresis loading buffer*

A pinch (0.25%) of bromophenol blue, a pinch (0.25%) of xylene cyanol, and 4g of sucrose (to make a final concentration of 40% sucrose) are added to 10 ml of 5X TBE

2.2.2.4 Cloning and sequencing

2.2.2.4.1 *LB (Luria-Bertani) medium*

The medium contained 1% tryptone, 0.5% yeast extract, 1% NaCl and has a pH of 7.0. To make up 1L volume, 10g tryptone (*Sigma*), 5 g of yeast extract (*Sigma*) and 10g of NaCl (Sigma) were dissolved in 950 ml of de-ionized water. The pH of the solution was adjusted to 7.0 with NaOH (*BDH*) and the volume brought up to 1L. The solution was autoclaved on liquid cycle for 20 minutes at 15 psi. The solution was allowed to cool to 55°C and 4 ml of ampicillin (5 mg/ml in 1M $MgSO_4$) (*Sigma*) and the solution was stored at 4°C.

2.2.2.4.2 *LB (Luria Bertani) agar*

LB-medium was prepared as described above, but 15g/L agar (*Sigma*) as added before autoclaving on liquid cycle for 20 minutes at 15 psi. After cooling and adding of ampicillin, and X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) [640 µl (50mg/ml in dimethyl formamide)] (*Promega*), the agar was poured into 100 mm plates

and left for about 20 minutes in a microbiological cabinet to harden. The plates were then stored at 4°C.

2.2.2.4.3 *"Blue dye mix" used for sequencing*

6µl Blue Dye (150mg/ml blue dextran, 25mM EDTA) and 72 µl Fresh Formamide (deionised) (*Sigma*) were added to a fresh tube. This was mixed with a pipette and vortexed for 10 seconds.

2.2.2.4.4 *3M sodium acetate (pH 5.2)*

40.83g of sodium acetate.$3H_2O$ was dissolved in 80ml of DDW. The pH was adjusted to 5.2 with 1 M HCl.

2.3 Antibodies

A wide range of murine monoclonal antibodies was used in the studies described in this thesis. The antibodies used and their specificities, dilutions, and sources are summarized in Table 2.1. The secondary antibodies and reagents used to detect the reactivity of primary antibodies are shown in Table 2.1.

Table 2.1. The characteristics of primary monoclonal antibodies used in immunohistochemistry

SPCIFICITY	CLONE	DILUTION	PRETREATMENT	SOURCE
CD21	1F8	1/50	PC 2 min	*DakoCytomation*
CD 3	Polyclonal anti-CD3	1/50	PC 2 mins	*DakoCytomation*
CD20	L26	1/400	MW pH 6	*DakoCytomation*
CD10	56C6	1/10	MW pH 9.9	*Novocastra*
BCL6	M7211	1/50 (IHC)	MW pH 9.9	*DakoCytomation*
CD57	NK-1	1/50	PC 2 mins	*Novocastra*
MUM-1/IR4	MUM1p	1/200	MW pH 9.9	*DakoCytomation*
CD4	1F6	1/200	MW pH 9.9	*Vector Labs*
CD8	C8/144B	1/50	MW pH 9.9	*DakoCytomation*
CD79a	JCB117	1/50	PC 2 min	*DakoCytomation*
CD23	1B12	1/10	PC 2 min	*Novocastra*
CD35	RLB25	1/40	MW pH 6	*Novocastra*
CD138*	5G10	1/50	MW pH 6	*Novocastra*
CD34	QBEnd/10	1/50	PC 2 min	*Novocastra*
CD30*	Ber-H2	1/20	PC 2 min	*DakoCytomation*
CD15*	C3D-1	1/10	PC 2 min	*DakoCytomation*
ALK-1*	ALK1	1/50	MW pH 9.9	*DakoCytomation*
CXCL-13**	53610	1/200	MW pH 6	*R&D Systems, Minneapolis, Minnesota, USA*
Ki 67	MIB-1	1/50	PC 3 min	*DakoCytomation*
LMP-1*	CS1-4	1/2000	PC 3 min	*DakoCytomation*
IgD*	Polyclonal anti-IgD	1/500	PC 3 min	*DakoCytomation*
Kappa*	Polyclonal anti-kappa	1/2000	MW pH 6	*DakoCytomation*
Lambda*	Polyclonal anti-lambda	1/2000	MW pH 6	*DakoCytomation*

*CD30, CD15, ALK-1, LMP-1, CD138, Kappa, Lambda,and IgD immunostaining was performed by the immunohistochemistry laboratory at University College London

**CXCL13 immunostaining was performed by the Pathology Department, Mayo Clinic, Rochester Minnesota.

Abbreviations: PC, pressure cooking; MW, microwave oven; min, minute

2.4 Tissues

Biopsies from 169 cases of nodal peripheral T-cell lymphoma were used in this study. Paraffin embedded tissue from all 169 cases and frozen tissue specimens from 5 cases were obtained from the surgical archives of Department of Histopathology, Royal Free and University College Medical School, London (137 cases), Departement de

Pathologie, CHU Henri Mondor, Creteil, France, Department of Histopathology (10 cases), The Royal Marsden NHS Foundation Trust, London (3 cases), Department of Pathology, Mayo Clinic, Rochester, Minnesota, USA (14), Servico de Anatomia Patologica, Instituto Portugues de Oncologia de Francisco Gentil, Lisbon, Portugal (2) and the Department of Pathology, Chi-Mei Medical Centre, Taiwan (2). Ethical approval was obtained for the use of archival tissues. The histology of all cases was reviewed by Professor Ahmet Dogan, Department of Histopathology, University College London. They were were categorized as follows: AITL (120 cases), PTLu (22 cases), ALCL (16 cases), AITL/PTL indeterminate (10 cases), nodal involvment by mycosis fungoides (1 case).

2.5 Methods

2.5.1 Immunohistochemistry

Immunohistochemical studies were carried out on paraffin-embedded tissue sections using protocols described below unless specified.

2.5.1.1 Single layered immunohistochemistry

Paraffin sections (4 μm) were cut from tissue blocks onto microscopic slides (*DakoCytomation*). These were de-paraffinized in xylene (*BDH*), rehydrated using decreasing concentrations of ethanol *(BDH)*, and incubated in peroxidase block solution for 10 minutes to block the endogenous peroxidase activity. Antigen retrieval was carried out prior to immunostaining. For optimisation, serial dilutions of the primary antibody were systematically tested, and the conditions that gave the best staining were used in subsequent experiments. The antigen retrieval methods used for different

antibodies are summarized in Table 2.1. Sections were then incubated with primary antibody at optimal dilution for 1 hour followed by biotinylated secondary antibody (ChemMate™ Link, Biotinylated Secondary Antibodies (AB2), and Streptavidin Peroxidase (ChemMate™ Streptavidin Peroxidase [HRP]) for 30 minutes, respectively. Finally, the staining was visualized with DAB in H_2O_2 and counter-stained with Mayer's haematoxylin (*BDH*). Throughout all immunohistochemistry procedures, the slides were washed in TBS-Tween (three times for 5 minutes each), between all incubation steps. For each antibody tested a positive control was used.

2.5.1.2 Double-layered immunohistochemistry

The pre-treatment method used had to be appropriate for both antibodies to be tested. In cases where CD10 staining was performed, the slides were always pre-treated using microwave retrieval (25 minutes) in pH 9.9 retrieval solution. The procedure of the first primary antibody was the same as that of immunohistochemistry on formalin-fixed and paraffin-embedded tissue sections. After development with DAB, slides the slides were rinsed with Tris buffered saline(TBS)/Tween for 2 × 5 minutes and were then incubated with the second primary antibody at an appropriate dilution for 3 hours. This was followed by application of the secondary antibody (AB2, ChemMate™ Link, Secondary Antibody) for 30 minutes. The latter was rinsed off by TBS/Tween followed by application of ChemMate™ APAAP Immunocomplex (APAAP) for 30 minutes. This was rinsed off by TBS/Tween and the staining was visualized with Fast blue (Vector® Blue Alkaline Phosphatase Substrate Kit III) substrate solution for 15 minutes (in the dark), rinsed in water and mounted with an aqueous mountant, as the fast blue reaction product is alcohol and xylene soluble. For each antibody demonstrated, a positive

control and negative control and a control for double staining were used. e.g. CD10/CD20 would need CD10/CD20, CD10/- and -/CD20.

Assistance with double-layered immunohistochemistry was provided by Ms Phillipa Munson, MSc

2.5.2 EBER (Epstein Barr virus encoded RNA) in situ hybridisation

Paraffin sections (4 μm) were cut from tissue blocks onto microscopic slides (*DakoCytomation*). These were de-paraffinized in xylene (*BDH*) 2 × 5 minutes.

In the mean time, an incubation tray and the Proteinase K buffer were placed in a 37 °C oven to keep warm and the Proteinase K (*Sigma*), probe (in-house PCR generated ssDNA EBER-probe) and hybridisation buffer were removed from freezer and kept at room temperature to thaw.

The de-paraffinized sections were re-hydrated as follows:

3 minutes in 95% ethanol [47.5 ml of ethanol (*BDH*) and 2.5 ml of DDW]

2 minutes in 70% ethanol [35 ml of ethanol (*BDH*) and 15 ml of DDW]

3 minutes in DDW

The sections were then rinsed in TBS for 3 minutes, and digested with 5μg/ml Proteinase K (Sigma) for 15 minutes at 37°C (500μl per slide).

Following the digestion, the sections were rinsed in TBS, 2 × 3 minutes, and then in ethanol, 2 × 3 minutes and air-dried for 7-10 minutes.

15 – 30 μl of the ssDNA EBER probe in hybridisation buffer (made up by mixing probe and hybridisation buffer in the following proportions: 1μl of probe to 50μl of hybridisation buffer) were applied on sections and covered with a cover-slip. The amount of probe required depended on the size of the section (e.g. 30μl, for 24 × 32 mm

cover-slip, and 15 µl for 22 × 22 mm cover-slip). Rubber cement was placed around the edges of the coverslip to prevent dehydration, and the slides were placed in the warm moist incubation tray in a 37°C oven overnight. Both 1 × SSC and 2 × SSC were kept warm at 37°C overnight.

Following overnight incubation, the rubber cement and coverslips were removed from the slides and the slides were placed in 2 × SSC for 20 minutes at 37°C and then 1 × SSC for 20 minutes at 37°C, following which the sections were rinsed in TBS/Tween for 3 minutes. The slides were then drained and alkaline phosphatase-labelled anti-digoxigenin *(Boehringer Mannheim, Germany)* diluted by adding 1/500 to 1/25 normal human serum (*Chemicon, Hampshire, UK*), was applied for 60 minutes. Following this, the sections were rinsed with TBS/Tween for 5 minutes and tipped off. NBT-BCIP chromogen was then applied and the sections were kept in the dark for 60 minutes. The slides were then washed in tap water and counterstained with 0.1% Nuclear Fast Red in 5% Aluminium Sulphate for 1 minute. After the staining was complete, the sections were dehydrated in alcohol series, cleared with xylene and mounted.

2.5.3 Double layered immunohistochemistry and EBER in situ hybridization (EBER-ISH)

EBER in situ hybridisation was performed as described previously in section 2.5.2 but not counterstained. The sections were then subject to immunohistochemistry by pre-treating the slides as needed and then immunohistochemistry performed using the ChemMate™ Detection Kit, Peroxidase/DAB, Rabbit/Mouse (*DakoCytomation [Code no: 5001]*). The method used was the same as described in section 2.5.1.1, except that the primary antibody was applied for 3 hours.

Assistance with double layered immunohistochemistry and EBER-ISH was provided by Ms Phillipa Munson, MSc.

2.5.4 PCR for T-cell receptor γ chain gene and immunoglobulin heavy chain gene framework 3 (FR3) PCR

2.5.4.1 DNA extraction from paraffin-embedded tissue sections

Methods for extraction of DNA from paraffin embedded material were evaluated and modified to suit the available material. The following method was employed for extraction of DNA from unstained and stained paraffin material:

One to five (depending on the size of the block) 5μm sections were cut from tissue blocks and placed into clean microtubes. Unstained or stained cut sections were scraped from slides, after removal of coverslips and mountant using xylene if necessary, and placed into microtubes. 400μl of xylene were added, the tubes mixed for 1-2 minutes and spun at full speed on a microcentrifuge for 5 minutes. The supernatants were removed using pipettes, 400 μl of absolute ethanol added to the pellets and the tubes mixed briefly. The tubes were spun at full speed for 5min and the supernatants removed as described above. The pellets were allowed to dry completely at room temperature before addition of 50-200μl proteinase-k/buffer. Digestion was carried out at 37°C overnight, or for three hours at 55°C after which the tubes were heated at 95°C for 5min to destroy proteinase-k activity. After briefly spinning, 1-5μl of the resulting solutions were used in the PCR reactions, or stored at -20°C.

2.5.4.2 Method for T-cell receptor γ chain gene and immunoglobulin heavy chain gene FR3 PCR

The following reagents were added to a final volume of 50μl:

5μl 10 × PCR buffer (*Promega,*), 200μM (4μM/μl) each dNTP (*Promega*) ,250ng (5ng/μl) each primer (see below), $MgCl_2$ *(Promega)* in an optimum concentration of 1.5 mM, 0.015 U/μl Platinum® *Taq* DNA polymerase (*Invitrogen Ltd, Paisley, UK*) and ultra-pure H_2O (*Sigma*) added to 45μl. 50-100ng high molecular weight DNA or 2-5μl of paraffin embedded tissue extract was then added into each tube with an added volume of ultra-pure water to make up a volume of 5 μl. This was overlayed with 50μl of mineral oil (*Sigma*). Tubes were spun briefly and placed on a thermal cycling machine [Phoenix thermal cycler (*Hybaid, Techne, U.K.*)] that was programmed to heat (hot start) to 95°C for 2 minutes. This was followed by 40 cycles of 93°C × 45 seconds (denaturation), 55°C for 45 seconds (annealing) and 72°C for 90 seconds (extension). After completion of 40 cycles, a final primer extension step at 72°C for 10min was carried out. Products (10μl) were run on 10% polyacrylamide minigels (section 2.5.4.3). All product analyses were performed in a separate room to PCR set-up and DNA extraction to reduce the risk of cross-contamination.

Primers targeting the T-cell receptor γ gene

Name	Sequence	Reference
VG-I	TCT GG (G/A) GTC TAT TAC TGT GC	(McCarthy *et al*, 1992)
VG-II	GAG AAA CAG GAC ATA GCT AC	(Goudie *et al*, 1990)
VG-III/IV	CTC ACA CTC (C/T)CA CTT C	(McCarthy *et al*, 1992)
JG-12	CAA GTG TTG TTC CAC TGC C	(McCarthy *et al*, 1992)
JPG-12	GTT ACT ATG AGC (T/C)TA GTC C	(McCarthy *et al*, 1992)

TCR-γ chain gene was amplified using the method described by McCarthy (McCarthy *et al*, 1992) using two reactions with primers VγI+VγII + VγIII/IV+ $J\gamma_{1/2}$ (product sizes approximately 70-95 base pairs) and VγI+ VγII + VγIII/IV+ $JP\gamma_{1/2}$ (product sizes 80-110 base pairs)

Primers targeting the immunoglobulin heavy chain gene

Name	Sequence	Reference
Ig-FR3	ACA CGG C(C/T)(G/C) TGT ATT ACT GT	(Trainor *et al*, 1990)
Ig-LJH	TGA GGA GAC GGT GAC C	(Trainor *et al*, 1990)

2.5.4.3 PAGE of T-cell receptor γ chain gene and immunoglobulin heavy chain gene FR3 PCR products

Minigel apparatus was used for PAGE of PCR products. The protocol for polymerisation of acrylamide (to make a 10% gel) was:

Reagents were mixed as follows- 13ml H_2O, 5ml Acrylogel 5 (Acrylamide: N-N`-Methylene bis-acrylamide 19:1) (*Sigma*), 2ml 10 × TBE, 200µl 10% ammonium persulphate (*Sigma*), 20µl TEMED (N,N,N,N -Tetramethyl-Ethylenediamine) (*Sigma*). The gel mixture was immediately loaded between the glass plates and well-forming combs slotted into place. After 15min the combs were carefully removed and the sample wells rinsed three times with 1 × TBE buffer. The apparatus was assembled according to the manufacturers recommendations, using 1 × TBE buffer. 10 µl of the PCR products were mixed with 2.5 µl of loading buffer before loading into wells. The gels were run at the appropriate voltage, usually 150V, for 45min- 1h. The gels were removed from the glass

plates, stained in ethidium bromide (1μg/ml) for 10-15min and viewed under ultra-violet light. Digital photographs were taken to provide a permanent record of the results.

2.5.5 Laser capture microdissection

Microdissection of CD10 positive cells and CD10 negative areas, CD57 positive cells, and EBER-ISH positive cells was performed by using PixCell II Laser Capture Microdissection system (Arcturus, Mountain View, CA), using 4-5-μm sections immunostained for CD10, CD57 or subject to EBER in situ hybridisation. The sections used were always recently stained/hybridised (within 24 hours of microdissection) and no mount was applied. They were dehydrated in increasing concentrations of alcohol that ended in absolute alcohol (ethanol – *BDH*).

A laser beam diameter of 7.5 mm was used to achieve single cell microdissection. For CD10 and CD57 stained sections, between 30 and 50 single cells were microdissected onto Capsure Transfer film (*Arcturus).* Extreme care was taken to prevent cross contamination during this procedure. The DNA was extracted by fitting the caps with microdissected material onto 0.5ml microtubes (which had their own caps cut off) containing 20 μl of proteinase K/buffer. The tubes were inverted and placed in a moist box and left at room temperature for 24 hours following which the proteinase K was inactivated by heating the tubes at 95°C for 5min. PCR for TCR-γ chain gene or IgH (FR3) gen was performed as described above in section 2.5.4.2. 5μl of extracted DNA was used for each PCR reaction, which was estimated to contain DNA from 6 CD10 positive /CD57 positive cells or a single EBER positive cell.

2.5.6 Cloning of PCR products

2.5.6.1 TA cloning vectors

The pGEM-T vector system (Promega) was used for the cloning of PCR products in chapter 3. In this system, the presence of single 3'-T overhangs at the insertion site ensures efficient ligation by taking advantage of the non-template dependent addition of a single deoxyadenosine to the 3'end of the PCR products by the thermostable Taq polymerase. T7 and SP6 RNA polymerase promoters flank the cloning region.

2.5.6.2 Ligation of PCR products into the pGEM-T plasmid vector system

PCR products were purified using QIA Quick Gel extraction Kit (*QIAGEN, West Sussex,* UK). Ligation of PCR products into TA cloning vectors was performed according to the manufacturer's instructions. 7µl of gel-purified PCR product were mixed with 1µl (50ng) of vector, 1 unit of T4 ligase (1 Weiss unit/ µl) and 1µl of T4 ligase 10 × buffer, and incubated for 3 hours at 15°C. At this point, a 3µl aliquot was removed for the transformation step. The remainder of the ligation reaction was allowed to incubate overnight at 4°C, so that the extended ligation reaction could be used as a back up if necessary.

2.5.6.3 Transformation of ligated PCR product:pGEM-T vector

Two duplicate sets of LB/ampicillin/X-gal plates were used for each ligation reaction. Manufacturer's instructions were followed and 2µl of ligated product: pGEM-T vector was added to a 1.5 ml microcentrifuge tube on ice. The JM109 competent cells (*Promega*) were removed from –70°C storage and allowed to thaw for about 5 minutes on ice and mixed gently. 50 µl of cells were aliquoted into each tube containing 2µl of

ligated product: pGEM-T vector. The tubes were gently mixed and placed on ice for 30 minutes and then heat shocked for 45 seconds at exactly 42°C. The tubes were then returned to ice for 2 minutes and 1ml LB broth was added to the tubes. The tubes were inverted to mix and then incubated for one hour at 37°C. The transformation culture was plated on duplicate plates (50 µl on one and 100 µl on the other) and incubated overnight at 37°C. Each white colony was transferred into 150µl of LB/Ampicillin broth using a micropipette tip and cultured at 37°C for 3 hours in a shaking incubator. The culture was then spun down and the supernatant discarded, 20 µl of PCR water added and the mixture was vortexed vigourously for 10 seconds. The mixture was centrifuged and the supernatant used as PCR template.

2.5.6.4 PCR of cloned products

PCR reaction of cloned products was set up as follows:

The total volume for each PCR reaction was 25µl. Each reaction contained 2.5 µl of 10 × PCR buffer (*Promega*) (final concentration of 1 ×), $MgCl_2$ *(Promega)* in an optimum final concentration of 1.6 mM, 100 µM each dNTP (*Promega*) final concentration and 0.5 µl of T7 (5 µM) primer *(Promega)*, 0.5 µl of SP6 (5 µM) primer (*Promega*) (final concentration of 0.2pmol of each primer), 0.1µl of Platinum Taq polymerase (5U/µl) *(Invitrogen)* (final concentration of 0.5pmol), 19.2 µl of ultra pure water (*Sigma*) and 1 µl of LB culture

The PCR reaction was set up to heat for 5 minutes at 94°C to breakup the cell membrane and release the recombinant DNA into solution. This was followed by 35 cycles of 94°C× 1 minute (denaturation), 55°C for 1 minute (annealing) and 72°C for 2 minutes

and a final extension step at 72°C for 10 minutes. The products were run on an agarose gel.

2.5.6.5 Verification of cloning vector amplification by agarose gel electrophoresis

Preparation of a 1% agarose gel was undertaken as follows:- 1g of agarose (*Sigma*) was dissolved in 100ml of 1 × TBE buffer (containing 0.1μg/ml ethidium bromide) by boiling. The agarose was poured into the gel apparatus and the comb added. When the gel was solid the comb was carefully removed and 1 ×TBE buffer added to cover the gel. 4-10μl of the PCR products were mixed with 1-2.5μl of loading buffer before loading into wells. The gels were run at 100-150V for 30min. The gels were removed from the gel apparatus and viewed under ultra-violet light. Amplification of products of the sizes predicted by addition of the insert to vector sequence was interpreted as successful cloning and sequencing was subsequently carried out.

2.5.7 Sequencing of cloned PCR products

The cloned PCR products were sequenced in both directions using ABI 377 DNA Sequencer.

2.5.7.1 Sequencing reaction

The final volume for each reaction was 10μl. 5 μl of each PCR product (approximately around 500ng total DNA) were mixed with 4 μl of dRhodamine Dye Terminator Mix (*Applied Biosystems, CA)* and 1μl of T7 primer (at 5μM concentration) or 1μl of SP6 primer (at 5μM concentration) (final concentration at 0.5pmol) – for sequencing in both directons.

After denaturation for 30 seconds at 96°C, each reaction mixture was amplified for 25 cycles as follows: 30 seconds at 96°C, 15 seconds of annealing at 50°C, and 1 minute of extension at 60°C.

2.5.7.2 Purification and precipitation of PCR products

PCR products were cleaned by precipitation with 100 µl of 80% ethanol and 6 µl of 3 M NaAc (pH 5.2) using a 96 well microtitre plate in the following manner. 100 µl of 80% ethanol and 6 µl of 3 M NaAc (pH 5.2) were added to each microtitre well and the well was placed on wet ice for 30 minutes. Following which, it was spun at 3000rpm for 1 hour. The plate was then tilted upside down on a paper towel to drain. After draining, the plate was placed on a new towel and pulse centrifuged for 5 seconds at 400 rpm. Following pulse centrifugation the plate was placed right way up and spun for 15 minutes to ensure that the pellet was not lost. The plate was then dried at 90^0C on a heat block for 2 minutes.

2.5.7.3 Sequence analysis

The PCR products were analysed on 4.5% polyacrylamide denaturing gels (Acrylamide: N-N`-Methylene bis-acrylamide 29:1) (*National Diagnostics, Hull, UK*) in 1×TBE buffer (*National Diagnostics*) using an ABI 377 automated fluorescent DNA sequencer (*Applied Biosystems, Foster City, California, USA*), which has a four-color detection system. The DNA pellet was mixed with 2µL blue dye. The mixture was denatured for 2 minutes at 94°C and 1.5µl was loaded into every alternate well on a prewarmed gel using membrane comb method according to Hamoudi and co-workers (Hamoudi *et al*, 2002). The gel was run for 8 hours at 150 watts power, 50 milli amps current, 1650 volts

voltage, scan rate of 1200 scans per hour and 50°C temperature. The labeled DNA fragments were separated during electrophoresis, the fluorescence was detected in the laser scanning region using filter set E. The data was collected and stored using the ABI Collection Software 2.0 (*Applied Biosystems)*. The fluorescent gel data collected during the run was automatically analysed by the DNA Sequencing Analysis software 3.0 (*Applied Biosystems*). Sample sheets were prepared using in house SampConvertor software. Sequence alignment was carried out using Sequence Navigator 1.0.1 (*Applied Biosystems*).

Assistance with sequence analysis was provided by Rifat A Hamoudi.

2.5.8 Quantitative (real-time) EBV-specific PCR on tumour tissues

The following was performed by Dr Yuan Ping Zhou.

2.5.8.1 DNA extraction

5~8 5μm sections cut from formalin-fixed and paraffin embedded tissues were de-waxed twice in xylene, washed twice in absolute ethanol, and then air-dried. Tissues were digested with proteinase K (2mg/ml) in 30mM Tris-HCl (pH 8.0) buffer containing 10mM EDTA and 1% sodium dodecyl sulfate at 55°C for 2 days, followed by purification of DNA with the Wizard genomic DNA purification method (*Promega)*. Briefly, the tissue digests were mixed with 1/3 volume of protein precipitation solution and centrifuged. The resulting supernatant was transferred into a fresh tube and sample DNA was precipitated with isopropanol, washed in 70% ethanol and air-dried. DNA pellet was dissolved in 50μl of 10mM Tris-HCl (pH8.0). Five frozen samples were also

similarly extracted from whole sections but digested for only 12 hours. The negative genomic control DNA and the EBV positive control DNA were prepared from normal peripheral blood lymphocytes and Namalwa cell line (diploid and carries 2 copies of EBV genome/cell) culture using a QIAGEN blood & cell culture DNA kit (*QIAgen, West Sussex UK*) respectively, which was confirmed by serial PCR screening for EBV. The DNA samples were quantified using GeneQuant pro (*Amersham pharmacia biotech, Cambridge, UK*).

2.5.8.2 Real-time PCR

Real time PCR was carried out to quantify EBV load in AITL samples using an iCycler iQ system (*Bio-Rad*) with SYBR Green I and iTaq. The primer pairs were designed from EBNA region for EBV copy number (product length: 106 bp), and from micro-globulin (B2M) region for cell number identification. (product length: 86 bp). 50ng of DNA was used as starting material for each reaction.

The optimum $MgCl_2$ concentration for PCR was 1.5 mM, and the thermocycler was programmed as follows: 94°C for 2 minutes followed by 2 cycles of 94°C × 30 seconds; 63°C~57°C each 45 seconds; 72°C × 30 seconds followed by 30 cycles of 94°C ×30 seconds; 56°C × 45 seconds; 72°C × 30 seconds. This was followed by an extension time of 7 minutes at 72°C

The primers were:

Primers	Sequence	Target
EBNA106-F	CCGGTGTGTTCGTATATGGAG	EBV EBNA
EBNA106-R	GGGAGACGACTCAATGGTGTA	EBV EBNA
B2M-b-F	TGCTGTCTCCATGTTTGATGTATCT	human Beta 2 Microglobulin
B2M-b-R	TCTCTGCTCCCCACCTCTAAGT	human Beta 2 Microglobulin

(Abbreviations: EBNA106-F, Epstein Barr virus nuclear antigen 106 forward; EBNA106-R, Epstein Barr virus nuclear antigen 106 reverse; EBV Epstein barr virus; B2M-b-F, β2 microglobulin-b-forward; B2M-b-R, β2 microglobulin-b-reverse)

2.5.8.3 Quantification of EBV

The conditions for real-time PCR were optimized prior to data collection. The standard curves were generated by serial dilutions of 1×10^6 copies/ul of both EBV and B2M extracted from Namalwa cell line (each cell contains 6.6 pg DNA and 2 copies of both EBV genome and B2M gene) (Figure 2.1). Once the experimental conditions had been optimized, the real-time PCR was performed in a 25 µl reaction mixture containing 12.5 µl SYBR Green Super-Mix (*Bio-Rad*), 200 nM of each sense and anti-sense primer, and 200 ng AITL sample DNA. All samples were amplified in triplicate. Real-time PCR of B2M was run in parallel for each sample. Melt-curve analysis was performed immediately after the amplification protocol for each case and only samples that showed specific amplification were included in the data analysis (Figure 2.1).

The virus copy number per 1000 cells was calculated after obtaining virus copies and B2M copies according to the related Ct (threshold cycle) values and the standard curves respectively as described previously (Junying *et al*, 2003).

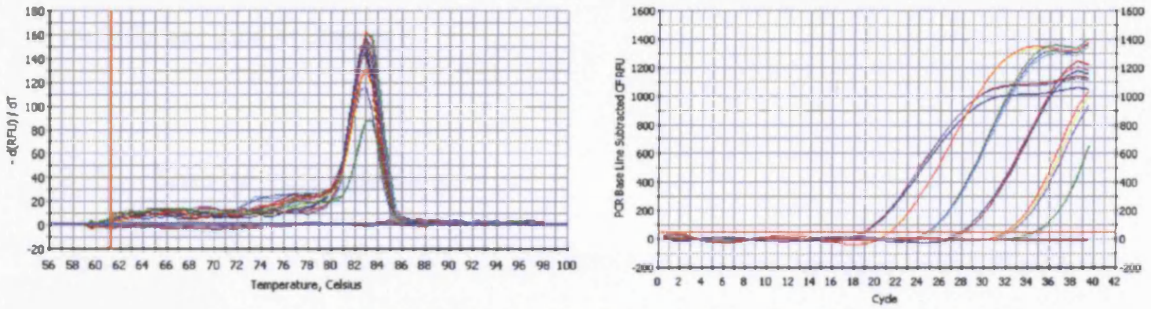

Figure 2.1 Melt curve analysis (left), and real-time PCR graph (right) for EBV

2.5.9 Conventional PCR for detection of HHV8

The following was performed by Dr Timothy C Diss:

The primers were novel and targeted a 200bp fragment of the putative minor capsid protein gene of HHV8:

Primers targeting the gene encoding a minor capsid protein of HHV*8*

Name	**Sequence**
KS-3	CCACCATTGTGCTCGAATC
KS-4	ACGATATGTGCGCCCCATAA

For HHV-8-specific PCR, the optimum $MgCl_2$ concentration for PCR was 1.5 mM, and the thermocycler was programmed as follows: 95°C for 2 minutes, followed by 40 cycles of 93°C × 45 seconds (denaturation), 61°C for 45 seconds (annealing) and 72°C for 90 seconds (extension). After completion of 40 cycles, a final primer extension step at 72°C for 10min was carried out.

2.6 Statistical analysis

Chi square (X^2) non-parametric test of statistical significance for bivariate tabular analysis was used in chapter 4.

2.7 Image processing

Images were visualized under an Olympus BX51 microscope equipped with UPlanFL x65, x40, x20, x10, x4 objective lenses and WH 10 x 22 eyepiece (Olympus, Tokyo, Japan). Images were captured with an Olympus DP70 camera and processed with Adobe PhotoShop 7.0 software (Adobe Systems, San Jose, California, USA).

Chapter 3

CD10 IS EXPRESSED BY THE NEOPLASTIC T-CELLS IN ANGIOIMMUNOBLASTIC T-CELL LYMPHOMA

3.1 Introduction

The diagnostic difficulties and the morphologic overlap of AITL with reactive conditions in early cases, and other lymphomas in those with less typical morphology have already been discussed in chapter 1. In this chapter we investigate for a possible phenotypic marker that would facilitate accurate diagnosis. The tumour cells, when identified in the form of clusters of clear cells have been reported to show a close association with the vascular proliferation that is typical of AITL (Nathwani & Jaffe, 1995). As the characteristic FDC proliferation in AITL shows a tendency to surround these vessels (Jaffe & Ralfkiaer, 2001a) it is not surprising that we noted a close association between the clear cell clusters, and the expanded FDCs and regressed follicular structures. Ree and co-workers (1998) and Kojima and co-workers (2001) describe cases of AITL showing hyperplastic GCs with ill-defined mantle zones, although the exact location of tumour cells in relation to these hyperplastic follicles is not clear. In peripheral lymphoid tissues expression of the antigen CD10 is restricted to the FDC/GC microenvironment and at the time of commencement of this project was believed to be confined to GC B-cells only (Arber & Weiss, 1997). In follicular lymphomas, CD10 expression is strongest within follicles and down-regulated in the interfollicular areas devoid of FDCs (Dogan *et al*, 1998), suggesting that the follicular/FDC micro-environment may play a role in CD10 expression. Although not

known to be a feature of mature T-cells or mature T-cell neoplasms, CD10 expression was described in 2 of 3 cases of T-cell lymphoma with a follicular growth pattern based on an FDC meshwork, described by de Leval and co-workers (2001). The possible influence of the FDC mico-environment on CD10 expression in mature lymphoid cells in turn raised the possibility that the close link between FDCs and the neoplastic T-cell in AITL may be associated with CD10 expression by the latter.

The main aim in this part of the project was to investigate whether the neoplastic T-cells in AITL express CD10. This was complicated because, unlike in many other lymphoid neoplasms where the tumour cells are present as a diffuse monomorphous proliferation, the infiltrate in AITL is typically polymorphic and the neoplastic T-cells are greatly outnumbered by and intimately admixed with reactive lymphocytes, histiocytes, plasma cells, eosinophils and FDCs (Jaffe & Ralfkiaer, 2001a;Willenbrock *et al*, 2005). Furthermore, clear cell clusters are not always present and cytologic features of malignancy may not be readily identifiable (Jaffe & Ralfkiaer, 2001a). On morphology alone, EBV infected transformed B-blasts could be mistaken for the neoplastic cells (Frizzera, 2001;Jaffe & Ralfkiaer, 2001a). It was therefore imperative that single cell analysis be undertaken to show proof of CD10 expression by neoplastic T-cells.

3.2 Results

3.2.1 Tissues used in this part of the project

Thirty formalin-fixed paraffin-embedded, lymph node biopsies from 30 cases of AITL were retrieved from departmental archives. The histology was reviewed and the diagnosis confirmed in each case. Of the cases of AITL, 4 were local cases, whereas the

remaining 26 cases were referrals from other centres for a second opinion. Details of the clinical presentation and the original diagnosis were obtained where possible.

3.2.2 Clinical features

These are summarised in Table 3.1. There were 17 males and 13 females between 37 and 84 years of age. Most patients presented with generalised lymphadenopathy. Other clinical features included weight loss, fever, skin rash, anaemia and hepatosplenomegaly. The initial histological diagnosis was AITL in only 13 cases. In the remaining 17 the diagnoses included reactive lymphoid hyperplasia (4 cases), T-cell-rich B-cell lymphoma (4 cases) and other lymphomas (8 cases).

3.2.3 Histology

The histological features are summarised in Table 3.2. The cases could be separated into three overlapping patterns.

In Pattern I (6 cases) there was partial preservation of the lymph node architecture. Hyperplastic B-cell follicles with poorly developed mantle zones and ill- defined borders were easily identifiable in the cortex of the lymph node (Figure 3.1A). These merged into the expanded paracortex containing a polymorphic infiltrate of lymphocytes, transformed large lymphoid blasts, plasma cells, macrophages and eosinophils within a prominent vascular network (Figure 3.1B and C). Increase in FDCs was not evident.

Table 3.1. Clinical features of AITL cases

Case No	A/S	Clinical features	Site	Initial diagnosis
1	65M	Fever, generalised lymphadenopathy, haemolytic anaemia	lymph node	AITL
2	62M	Generalised lymphadenopathy	lymph node	PTL
3	59M	Fever, cervical lymphadenopathy, abnormal liver function tests	lymph node	AIL
4	37M	Generalised lymphadenopathy	inguinal lymph node	Reactive
5	82M	Generalised lymphadenopathy, splenomegaly, coomb's test positive, night sweats	lymph node	Reactive
6	59F	Cervical lymphadenopathy, pleural effusion	lymph node	DLBL
7	62F	Lymphadenopathy, polyclonal gammopathy, hepatosplenomegaly	lymph node	AIL
8	70M	Generalised lymphadenopathy, weight loss, rash	axillary lymph node	HL, TRBL
9	48M	Not available	lymph node	AITL
10	64F	Generalised lymphadenopathy, fever, pruritus, night sweats	axillary lymph node	AIL?, reactive?
11	76F	Generalised lymphadenopathy, hepatosplenomegaly	lymph node	AIL?, CD?, reactive?
12	79F	Not available	lymph node	AITL?, PTL?
13	84F	lymphadenopathy, rash	inguinal lymph node	PTL?, TRBL?
14	78M	Fever, generalised lymphadenopathy, splenomegaly, skin rash	lymph node	NHL
15	42M	Generalised lymphadenopathy, fever, hepatosplenomegaly, haemolytic anaemia, weight loss	lymph node	Reactive?, CD?
16	60F	Fever, bilateral inguinal lymphadenopathy, splenomegaly	inguinal lymph node	NHL
17	58F	Generalised lymphadenopathy, "B" symptoms	lymph node	DLBL
18	78M	Generalised lymphadenopathy, coomb's positive haemolytic anaemia, hypergammaglobulinaemia,	cervical lymph node	AIL
19	55M	Generalised lymphadenopathy, fever, weight loss	inguinal lymph node	Reactive
20	71M	Inguinal lymphadenopathy, weight loss	inguinal lymph node	DLBL
21	39F	Generalised lymphadenopathy, night sweats	lymph node	AITL
22	77F	Generalised lymphadenopathy, splenomegaly, "B" symptoms	axillary lymph node	PTL?, HL?
23	52F	Lymphadenopathy, and high ESR	cervical lymph node	DLBL?, HL?
24	61M	Axillary, inguinal lymphadenopathy, hepatomegaly, pneumonia	axillary lymph node	AIL
25	61M	Not available	lymph node	AITL
26	74F	Generalised lymphadenopathy, pruritus	cervical lymph node	AIL
27	78F	lymphadenopathy, "B" symptoms	inguinal lymph nde	TRBL
28	78M	Generalised lymphadenopathy, rash, weight loss	inguinal lymph node	TRBL?, PTL?
29	52M	Generalised lymphadenopathy, hepatosplenomegaly, anaemia	lymph node	FDC tumour
30	59M	Generalised lymphadenopathy	lymph node	AIL

A: age, S: sex, M: male, F: female, AITL: angioimmunoblastic T-cell lymphoma, PTL: peripheral T-cell lymphoma, AIL: angioimmunoblastic lymphadenopathy, DLBL: diffuse large B-cell lymphoma, HL: Hodgkin's lymphoma, TRBL: T-cell rich B-cell lymphoma, : non-Hodgkin's lymphoma, CD: Castleman's disease FDC: follicular dendritic cell

Pattern II (9 cases) was characterised by loss of normal architecture except for the presence of occasional depleted follicles with concentrically arranged FDCs (Figure 3.2A-C). In some cases FDC proliferation extending beyond the follicles could be identified (Figure 3.2D). The rest of the node showed a polymorphic infiltrate with increased numbers of transformed lymphoid blasts and vascular proliferation similar to that described for pattern I (Figure 3.2D-F).

In pattern III (15 cases) the normal architecture was completely effaced and no B-cell follicles could be identified. Prominent irregular proliferation of FDCs could be seen in H&E stained sections in most cases and this was accompanied by extensive vascular proliferation and a polymorphic infiltrate similar to that seen in patterns I and II (Figure 3.4A, G and H).

In 16 cases perivascular collections of cells with clear cytoplasm were evident (Figure 3.2F).

3.2.4 Immunohistochemistry

In pattern I sprouts of CD21 positive FDCs extended beyond the confines of the CD21 positive GCs and occasionally enveloped small blood vessels (Figure 3.1D). Paracortical T-cells, including some transformed blasts expressed CD3 (Figure 3.1F) and predominantly CD4. A smaller CD8 positive population was present. Preserved GCs expressed CD20 and CD10. IgD highlighted the mantle zones (Figure 3.1E). In addition to CD10 positive GC cells there was a population of smaller strongly staining lymphoid cells at the outer rim of the GC that extended into the paracortex (Figure 3.1G-I).

In pattern II, CD21 positive FDCs extended beyond the follicles into the paracortex often surrounding proliferating small vessels (Figure 3.2G).

In Pattern III they showed a more haphazard arrangement, surrounding the arborising small vessels (Figure 3.4B, I and L).

In both patterns II and III, CD20, IgD positive but CD10 negative clusters of small B-cells, mostly not associated with GCs, could be identified within meshworks of CD21 positive FDCs. In all but 3 cases (4 lymph node biopsies) there were concentrations of CD10 positive T-cells around the CD20 positive B-cell clusters as described for pattern I (Figure 3.3D-F, Figure 3.4C,F, J and K). In addition isolated CD10 positive T-cells were scattered throughout the entire lymph node and formed perivascular aggregates corresponding to the clear cells noted in H&E stained sections (Figure 3.3C and F). CD10 was also expressed by granulocytes most of which were within blood vessels. Their characteristic cytological features easily distinguished them from the CD10 positive lymphoid cells. Clear cells when present were CD4 positive (Figure 3.2J, left panel) and CD8 negative (Figure 3.2J, right panel). In biopsies showing patterns II and III isolated transformed CD20 positive blasts were identified within the predominantly CD3 positive infiltrate.

3.2.4.1 Sequential double staining

Double staining with CD20 followed by CD10 was performed on all lymph node biopsies to show that the lymphoid cells expressing CD10 were not GC B-cells. In 27 out of 30 biopsies (27 of 30 cases) there were varying numbers of CD20 negative, CD10 positive lymphoid cells strongly suggesting that these were T cells (Figure 3.1, Figure 3.3 G-I and Figure 3.4D and E). Double staining with CD79a and CD10 gave identical

results. The T-cell phenotype of the CD20 negative, CD10 positive lymphoid cells was also confirmed by CD3/CD10 double staining which showed a population of "purple" cells co-expressing CD3 and CD10. The ratio of CD10 positive T-cells to all T-cells for each case is shown in Table 3.2.

The approximate ratio of CD10 positive T-cells to all T-cells was assessed from serial sections immunostained for CD3 and CD20/CD10. Further double staining experiments showed that the CD10 positive T-cells expressed CD4 but not CD8. Although the proliferation fraction as assessed by single layered Ki 67 staining was high (Figure 3.1O and Figure 3.2K), double staining of the CD10 positive cells with Ki-67 showed that these phenotypically aberrant T-cells had a low proliferation fraction (Table 3.2 and Figure 3.2L). The proliferation fraction was calculated by counting 500 CD10 positive cells in 5-7 different areas of the lymph node on sections double immunostained for Ki67/CD10.

3.2.5 In-situ hyridisation for EBV-EBER

All cases of AITL showed hybridisation for EBV-EBER in a subset of the cells. The number of cells expressing EBV-EBER varied markedly from case to case, in some only scattered cells were positive, in others numerous large blasts were labelled. The distribution of EBER positive cells was similar to the distribution of large B-blasts and did not correspond to the clusters CD10 positive T-cells.

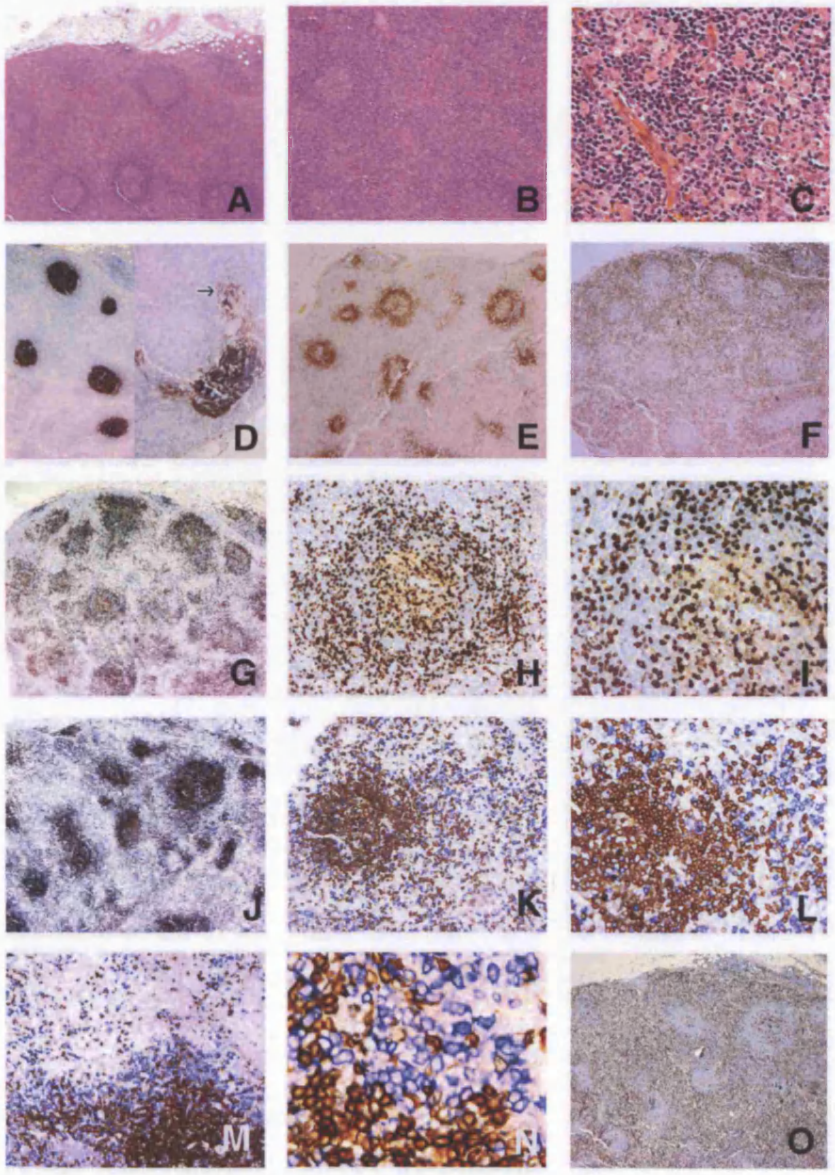

Figure 3.1. Case 2 showing "Pattern I" histology with hyperplastic follicles.

(A-C) Haematoxylin and eosin stained section showing hyperplastic follicles (A, B) and a polymorphous infiltrate associated with prominent vascularity in the paracortex (B,C). (D) CD21 stain shows that there is no obvious (left panel), or subtle expansion (right panel) of the follicular dendritic cell meshwork. (E) IgD highlights the mantle zones. (F) CD3 highlights numerous T-cells.(G-I) CD10 highlights many lymphoid cells which include the lighter staining GC B-cells and T-cells which show crisp, dark staining. (J-M) CD20/CD10 double staining shows many CD10 positive (blue) cells that are negative for CD20 (brown), consistent with T-cells. (O) Ki-67 staining shows a high proliferation fraction.

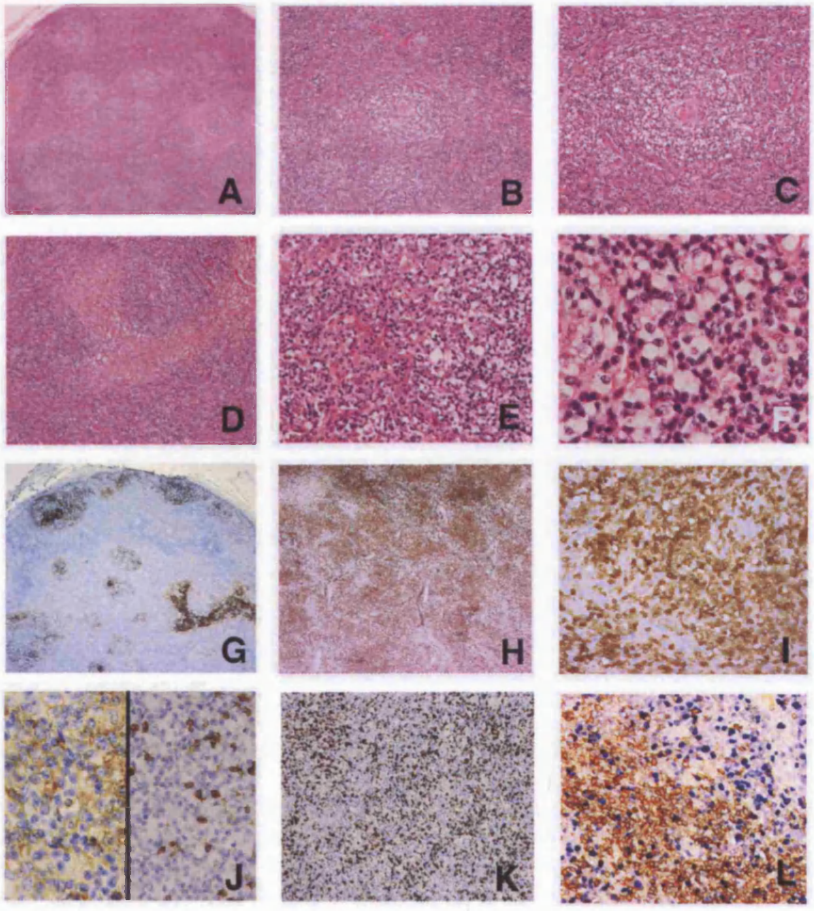

Figure 3.2 Case 10 showing "pattern II" histology.

(A-F) Haematoxylin and eosin stained section showing a vague nodularity (A) with regressed follicles associated with clusters of clear cells (B and C). Fasicles of pale cells comprise the hyperplastic follicular dendritic cell (FDC) meshwork (D). A rich vascular network is intimately associated with the clear cells (E). Panel F shows the clear cells at high power. (G) CD21 stain highlights the hyperplastic FDC. (H,I) CD3 stain shows numerous T-cells, including large clear cells. (J) The clear cells are CD4 positive (J, left panel), and CD8 negative (J, right panel). (K) Ki 67 shows a high proliferation fraction. (L) CD10/Ki 67 stain shows that most CD10 positive cells (brown) are Ki 67 (blue) negative.

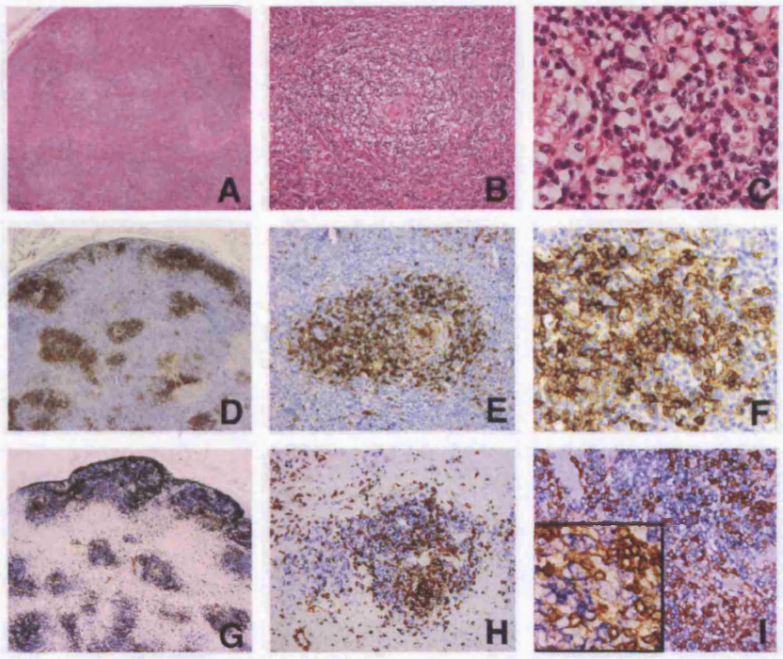

Figure 3.3 Case 10 showing CD10 positive T-cells that correspond to the clear cells.

Panels A-C show low, medium and high power views of haematoxylin and eosin (H&E) stained section while panels D-F, show CD10 immunostaining and panels G-I show CD20/CD10 double staining at similar magnifications. CD10 positive lymphoid population (D-F) correspond to clear cells seen on H&E staining (A-C). CD20/CD10 staining shows many CD10 positive cells (blue) that are CD20 (brown) negative, consistent with T-cells (G-I). These also correspond to the clear cell population seen on H&E (A-C).

3.2.6 PCR for TCRλ and IgH gene re-ararrangement

The results of PCR for TCRγ gene and IgH gene are shown in Table 3.2 and Figure 3.6. There was a monoclonal (1-2 dominant bands) or oligoclonal (3-4 dominant bands) T-cell population in all cases except one. Five of the cases also showed evidence of a monoclonal B-cell population. In all of these cases numerous EBV infected transformed lymphocytes were present.

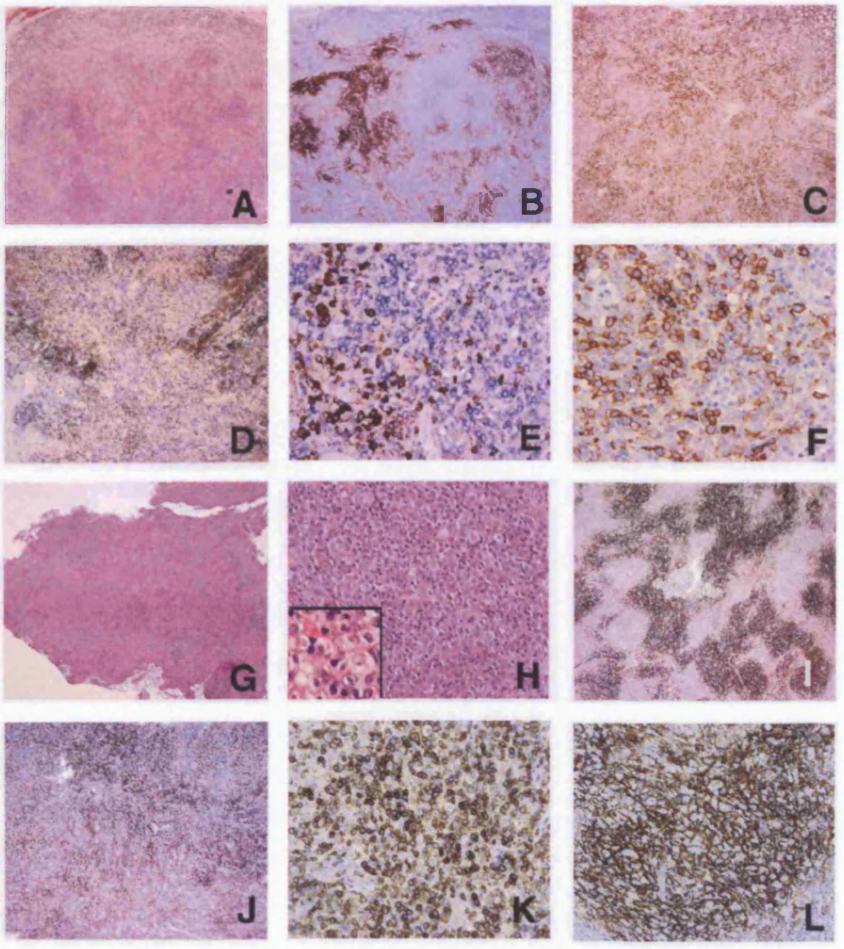

Figure 3.4 Cases 28 and 27 showing pattern III histology and CD10 positive T-cells.

Panels A-F show sections from case 28 and panels G-L show sections from case 27.
(A, G, H) Haematoxylin and eosin stained sections showing complete effacement of architecture with no identifiable follicles, prominent vascularity (H) and clear cells (H, inset). (B ,I and L) CD21 shows marked hyperplasia of the follicular dendritic cell meshwork, which shows a tendency to surround vessels (L). (C,F, J and K) CD10 stain shows numerous CD10 positive lymphoid cells. (D and E) CD20/CD10 double staining shows numerous CD10 positive (blue) lymphoid cells that are CD20 (brown) negative, consistent with T-cells

Table 3.2. Summary of histological features, immunophenotype and molecular analysis of AITL

Case No	Pattern*	CD10/CD3†	Clear cells‡	CD10/Ki67§	TCR-PCR¶	IgH-PCR¶
1	1	5%	absent		O	P
2	1	5%	absent	16%	M	P
3	1	10%	present		O	P
4	1	10%	absent		M	P
5	1	20%	present	8%	M	P
6	1	0%	absent		M	P
7	2	5%	absent		P	P
8	2	5%	absent		M	P
9	2	5%	absent	11%	M	P
10	2	10%	present	19%	O	P
11	2	10%	present		M	M
12	2	15%	present		M	P
13	2	20%	absent		M	M
14	2	20%	absent		O	M
15	2	5%	absent		M	P
16	3	0%	absent		M	P
17	3	5%	present		M	P
18	3	5%	present		M	P
19	3	5%	present		M	P
20	3	5%	present		O	M
21	3	10%	absent		M	P
22	3	10%	absent		M	P
23	3	20%	present		M	P
24	3	20%	present		M	NA
25	3	20%	present	10%	M	P
26	3	30%	absent	11%	O	P
27	3	30%	present	19%	M	M
28	3	30%	present		M	P
29	3	30%	present		O	P
30	3	0%	present		M	P

* The histological pattern as described in the results. ** categorized as pattern II † Percentage of CD10 positive and CD3 positive T-cells. ‡ Cases containing aggregates of atypical lymphoid cells with clear cytoplasm. § Percentage of CD10 positive cells expressing Ki67. ¶ Results of PCR performed for T-cell receptor gamma chain gene (TCR) and immunoglobulin heavy chain gene (IgH). M: monoclonal, O:oligoclonal, P:polyclonal, NA: no amplification

3.2.7 Single cell microdissection

To investigate whether the CD10 positive T-cells were part of the neoplastic clone, 30-50 CD10 positive cells were microdissected from 5 cases (Figure 3.5) and PCR for TCRγ gene was performed. In each case analysis of the PCR products from microdissected cells gave a dominant band/s identical in size to that was observed for the PCR performed on whole sections. (Figure 3.6) In contrast DNA extracted from areas lacking CD10 positive cells showed a polyclonal ladder or smear.

3.2.8 Cloning and sequencing of PCR products

The cloning and sequencing of the dominant bands isolated from PCR products of three cases showed a dominant clone with identical sequence, confirming the monoclonal nature of all cases examined. For each of the three cases the dominant clone from the whole sections and from the microdissected CD10 positive cells were identical (Table 3.3 and Figures 3.7 and 3.8).

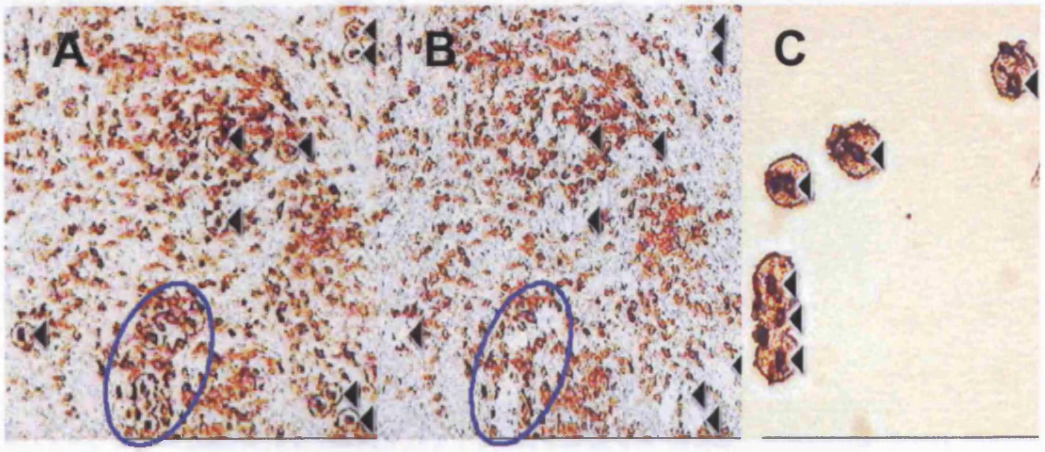

Figure 3.5. Laser capture microdissection of CD10 positive lymphoid cells in AITL.
(A) CD10 stained section before microdissection; the cells targeted for microdissection are indicated within the blue circle and by arrowheads. (B) Same area after microdissection; the blue circle and arrowheads show the spaces left after cells have been removed. (C) High-power view of a cluster of CD10 positive cells after microdissection. These are the encircled cells/spaces in panels A and B.

Table 3.3: Results of cloning and sequencing of dominant bands obtained from PCR amplification of TCR γ chain gene from whole lymph node sections and microdissected CD10 positive cells

Case No	PCR band	Whole lymph node*	Microdissected CD10 positive cells †
12	Upper	4/6	6/6
	Lower	3/6	6/6
26	Upper	3/5	4/6
	Lower	11/13	8/8
28	Upper	7/9	5/7
	Lower	10/10	Not done

* The number of clones with identical TCR γ chain gene sequence / total number of clones sequenced.

† The number of clones with TCR γ chain gene sequence identical to the dominant sequence obtained from the whole section PCR / total number of clones sequenced.

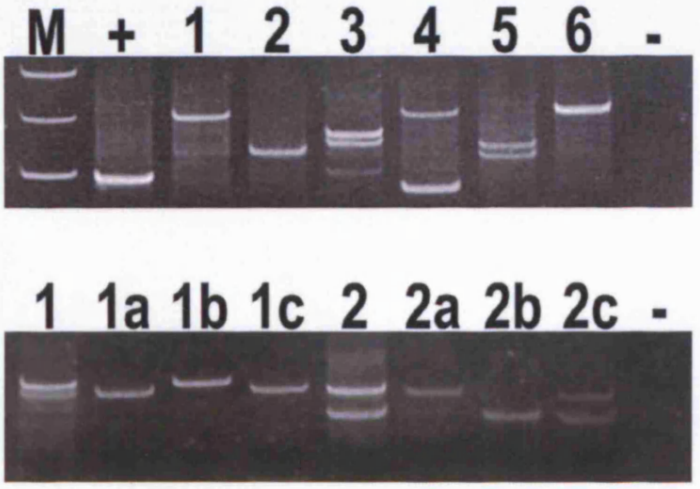

Figure 3.6 Analysis of PCR products for TCR-γ chain gene on polyacrylamide gels in AITL.

(Top panel) All cases demonstrated in the figure show 1 or 2 dominant bands consistent with a monoclonal T-cell population. Lane M, molecular weight markers; lane 1, positive control; lane 1, case 21; lane 2, case 23; lane 3, case 26; lane 4, case 22; lane 5, case 3; lane 6, case 4; and lane 2, negative control. (Bottom panel) Microdissection of CD10 positive cells in cases 26 and 13 gave PCR products identical in size to products obtained from whole-section PCR. Lane 1, whole-section PCR products of case 26; lanes 1a,b,c, microdissected PCR products of case 26; lane 2, whole section PCR products of case 13; lanes 2a,b,c, microdissected PCR products of case 13; and lane 2, negative control.

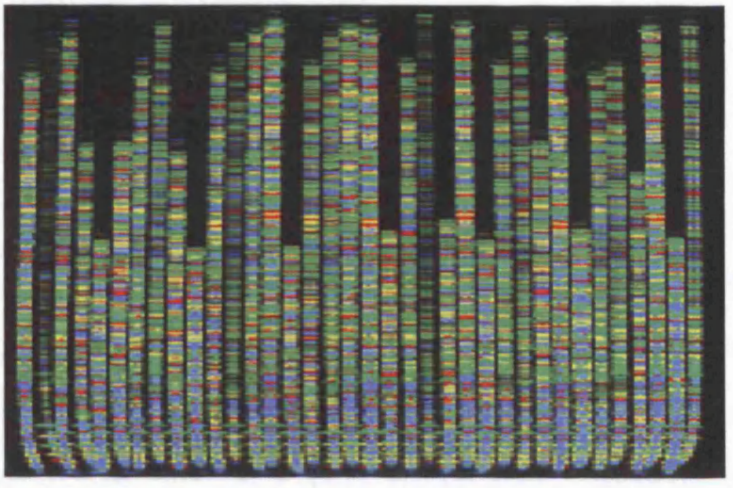

Figure 3.7 Gel image of TCR γ chain gene sequences

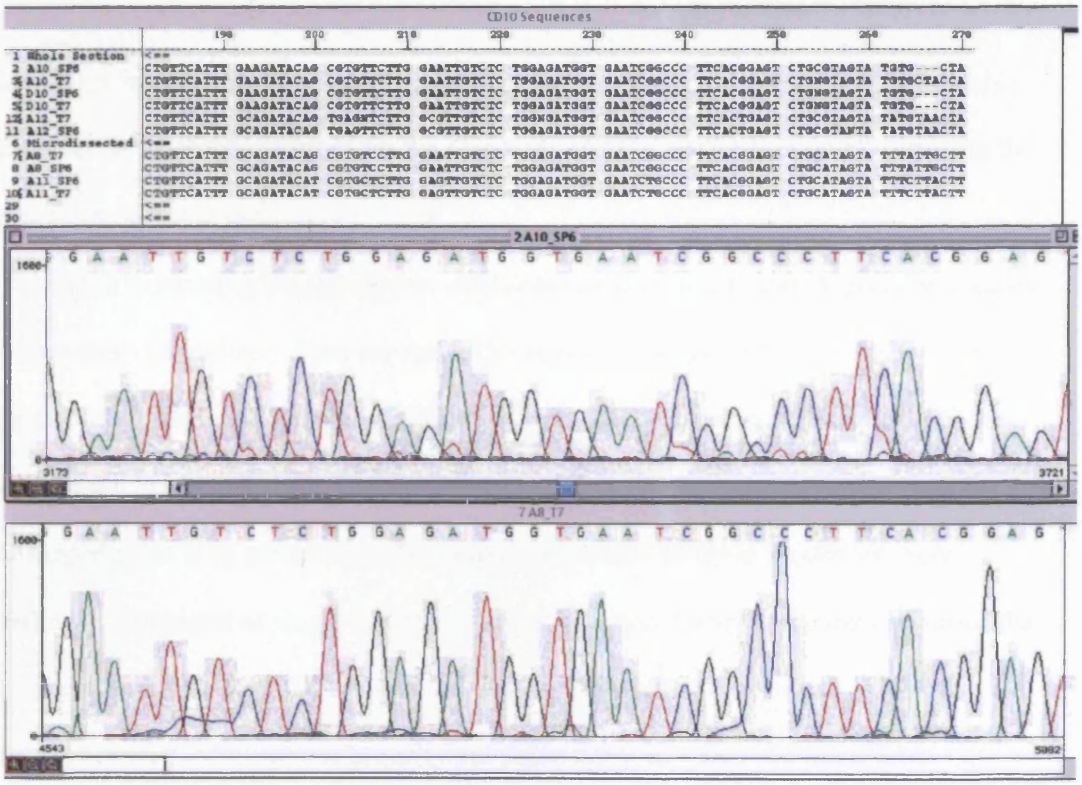

Figure 3.8 DNA sequence alignment of rearranged TCR γ chain gene from whole lymph node section (upper panel) and microdissected CD10 positive cells (lower panel).

3.3 Discussion

3.3.1 CD10 expression in AITL

The histological diagnosis of AITL may be difficult as is shown by the large error rate (13/26; 50%) in referred cases in our series. This is especially true when preservation of follicles and only slight expansion of the paracortex are present. The expanded CD21 positive FDC meshwork is very helpful in making the diagnosis (Leung *et al*, 1993) but can be subtle. Molecular investigation and, in many instances repeated biopsies may be required to reach a confident diagnosis. In this study we have shown that CD10 is a

phenotypic marker that specifically identifies the tumour cells in 90% of AITL including, importantly, the "early" cases with pattern I histology. We believe that this provides an objective criterion for the diagnosis of AITL and should greatly assist in the diagnosis of this disorder.

The CD10 expressing T-cells must be distinguished from GC B-cells especially in cases with pattern I histology. They are typically seen at the margins of B-cell follicles as small to medium sized lymphoid cells with crisp membrane staining in contrast to the larger follicle centre B-cells that stain more diffusely and with less intensity. In more advanced cases with pattern II or III histology the CD10 positive T-cells are more diffusely distributed as single cells and in small clusters. Double staining confirmed that these cells are CD3 and CD4 positive T-cells that accounted for a relatively small fraction of all T-cells present in each case. Although it was believed that CD10 expression is not a feature of normal / reactive mature T-cells, this has recently been challenged by Cook and co-workers (Cook *et al*, 2003) who demonstrate a small subset of benign CD10 positive T-cells detected by flow cytometry in 5 of 28 cases (18%) of reactive lymphoid hyperplasia, 4 of 17 (23%) of follicular lymphoma and 9 of 19 cases (47%) of marginal zone B-cell lymphoma. Using double layered immunohistochemistry with CD10 and PAX-5, the latter, a lineage specific B-cell marker that stains the nucleus, they showed that many of the CD10 positive, PAX-5 negative presumptive T-cells were located in the follicle centers. Despite these findngs, several lines of evidence point to the CD10 positive T-cells in AITL being neoplastic rather than reactive. The clusters of large cells with clear cytoplasm, which occur in some cases and widely regarded to represent the neoplastic clone in AITL (Frizzera, 2001;Nathwani & Jaffe, 1995), were consistently CD10 positive. Most compelling and the ultimate proof is that

clonal analysis following micro-dissection has shown that the neoplastic clonally rearranged TCR genes are confined to the CD10 positive population.

A subset of AITL cases contains more than one dominant T-cell clone with molecular or cytogenetic studies (Feller *et al*, 1988;Schlegelberger *et al*, 1990a;Schlegelberger *et al*, 1994b;Smith *et al*, 2000). Although we did not specifically address whether all dominant clones in a given case expressed CD10, all AITL cases with an oligoclonal T-cell population contained CD10 positive T-cells suggesting that at least one of the dominant clones expressed CD10.

The reasons for the absence of CD10 positive T-cells in the small minority of cases (3/30 in the present series) that were otherwise entirely typical of AITL are unclear. Occasionally cells that normally express CD10, such as GC B-cells, fail to do so (Unpublished observations). Sometimes this is due to technical reasons, such as the fixation method, but more often it appears that the antigen has been down-regulated. The reasons for this currently remain obscure.

The cases with the histological patterns II and III fulfill the conventional criteria for the diagnosis of AITL. Thus there was clinical evidence of a systemic disease characterised by generalised lymphadenopathy, hepatosplenomegaly and anaemia together with characteristic histological features in lymph node biopsies. These include effacement of lymph node architecture by a polymorphic infiltrate of lymphoid cells, proliferation of FDCs and small vessels, the presence of varying numbers of EBV infected transformed B-cells, and the presence of clusters of large transformed cells with clear cytoplasm. In addition there was molecular evidence for the presence of monoclonal or oligoclonal T-cell populations. Whether the cases falling into histological pattern I can also be considered within the same spectrum is more controversial. The presence of hyperplastic

follicle centres in otherwise typical AITL was first described by Ree and co-workes (Ree *et al*, 1998) who reported progression of two such cases into typical AITL. We observed the CD10 positive T-cells in all histological patterns including the cases with hyperplastic GCs. Pattern I cases had the smallest number of CD10 positive T-cells whilst pattern III cases had the most. These findings suggest that all three histological patterns of AITL described are part of the same disease process and the histological patterns I, II and III form a biological continuum.

In five cases where microdissection was performed only the CD10 positive T-cells were clonal and we failed to detect the presence of the clonal T-cells in microdissected CD10 negative areas. Thus, the proportion of neoplastic T-cells in AITL would appear to be small (5%-30%) and it appears most of the lymph node enlargement is accounted for by reactive cells similar to what occurs in Hodgkin's lymphoma. This was also shown by Willenbrock, *et al* (2005) using triple immunofluoresent staining with antibodies directed against the T-cell receptor β–family specific epitopes and single cell PCR analysis. Their observations were that in some cases of AITL, the tumour cells comprised a minority of the T-cells present. This was also the conclusion of Schegelberger and co-workers (Schlegelberger *et al*, 1994b) when on interphase cytogenetics, the percentage of cells with cytogenetic abnormalities formed a small proportion of all cells.

3.3.2 CD10 / neutral endopeptidase EC 3.4.24.11 (NEP)

CD10 in haemaopoeitic tissues was identified by the name "common acute lymphoblastic leukaemia antigen (cALLA)" which was originally recognized by using a hetero-serum developed in rabbits by immunizing them with malignant lymphoblasts

obtained from a case of "non B, non T-cell" acute lymphoblastic leukaemia (Greaves *et al*, 1975). Following this discovery, monoclonal antibodies that recognized cALLA were developed and were clustered as CD10 at the First Leukocyte Differentiation Workshop (Bernard *et al*, 1984;Ritz *et al*, 1980). CD10 or neutral endopeptidase EC 3.4.24.11 (NEP), is a cell surface zinc metallo-proteinase that is expressed by subsets of lymphoid cells, and also by terminally differentiated granulocytes, but not immature myeloid cells, and a variety of non-haemopoietic cells such as bronchial epithelial cells, renal proximal tubular cells, endometrial stromal cells, breast myoepithelial cells, and fetal intestine among others (Baraniuk *et al*, 1995;McCluggage *et al*, 2001;McIntosh *et al*, 1999;Moritani *et al*, 2002;Shipp & Look, 1993;Skrzydlo-Radomanska *et al*, 1993;Sont *et al*, 1997;Trejdosiewicz *et al*, 1985). CD10/NEP is also widely expressed in a variety of non-haematopoietic neoplasms including renal cell carcinoma (~90%), endometrial stromal sarcoma (85-100%), transitional cell carcinoma of the genito-urinary tract (54%) and prostatic carcinoma (61%) (Avery *et al*, 2000;Chu & Arber, 2000;Chu *et al*, 2001;Langner *et al*, 2004;McCluggage *et al*, 2001;Song *et al*, 2004;Toki *et al*, 2002;Zhu *et al*, 2004). It may also be expressed by pancreatic adenocarcinoma, malignant melanoma, rhabdomyosarcoma and schwannoma among others (Bilalovic *et al*, 2004;Chu & Arber, 2000;Jongeneel *et al*, 1989). In lymphoid cells, CD10 is restricted in its expression. In precursor lymphoid cells it is expressed by both precursor B- and T-cells, whereas in mature lymphoid cells its expression is limited to the follicle center compartment.

In the bone marrow, the CD10 positive lymphoid cells include progenitors uncommitted to the B-or T-cell lineage, TdT positive, CD34 positive, CD19 positive B-cell precursors (Hokland *et al*, 1984;LeBien *et al*, 1990;Loken *et al*, 1987) and also a significant

proportion of T-cell precursors (Gore *et al*, 1991). The percentage of CD10 positive lymphoid cells in the marrow decreases with age, from 13 to 40% in the fetus (Delia *et al*, 1985;Gore *et al*, 1991;LeBien *et al*, 1990) to 1% in the elderly (Gore *et al*, 1991). In the fetus almost all bone marrow B-cells are CD10 positive (Punnonen *et al*, 1992). The proportion of CD10 positive (also TdT positive) lymphoid cells in the marrow increases in a regenerating marrow and following transplantation (Kobayashi *et al*, 1991).

In the thymus, some normal CD34 expressing immature T-cells also express CD10. The expression is strongest in immature T-cells that are CD3 negative/weakly positive and CD4- and CD8-, moderate in those that are CD4, CD8 double positive or CD4-, CD8 positive and weak or absent in those that are CD4 positive, CD8- (Mari *et al*, 1994).

In peripheral lymphoid tissues, CD10 expression is confined to the follicle center compartment and is a feature of follicle center B-cells (Arber & Weiss, 1997;Barcus *et al*, 2000;Dogan *et al*, 2000) and a small subset of follicle center T-cells, the characterization of which, including their functional properties are yet to be studied (Cook *et al*, 2003).

In lymphomas, CD10 is expressed in 90% of precursor B-lymphoblastic lymphoma / leukaemia and (27-40%) precursor T-lymphoblastic lymphoma/leukaemia (Arber & Weiss, 1997;Boucheix *et al*, 1994;Chu & Arber, 2000;Dowell *et al*, 1987;Pui *et al*, 1993). High levels of CD10 expression in precursor B- and T-lymphoblastic lymphoma / leukaemia is associated with a better prognosis (Basso *et al*, 1992;Boucheix *et al*, 1994;Kersey *et al*, 1982). In peripheral lymphoid neoplasms CD10 expression is confined to B-cell lymphomas (Arber & Weiss, 1997) and is a feature of the majority (90%) of follicular lymphomas, and an almost consistent feature of Burkitt lymphoma. (Dogan *et al*, 2000;Harris *et al*, 1994;Harris *et al*, 2000) It is also seen in approximately

30% of BLBCLs, many of which may represent transformed follicular lymphomas (Dogan *et al*, 2000;Ohshima *et al*, 2001). CD10 is also expressed by a small number of mantle cell lymphomas (Dong *et al*, 2003;Xu *et al*, 2002b) and 10 – 26% of hairy cell leukaemias (Jasionowski *et al*, 2003;Robbins *et al*, 1993). It has also been reported in a small proportion of plasma cell myelomas, a feature that is reported to determine a poorer outcome (Durie & Grogan, 1985). CD10 expression is very unusual in other small B-cell neoplasms such as small lymphocytic lymphoma / chronic lymphocytic leukaemia, lympho-plasmacytic lymphoma, nodal marginal zone lymphoma and splenic marginal zone lymphoma (Arber & Weiss, 1997).

Except for a single report of 2 cases of peripheral T cell lymphomas (PTL) with a follicular growth pattern and CD10 expression (de Leval *et al*, 2001), the latter has not been a feature of mature T-cell and NK-cell neoplasms. In fact, these 2 CD10 positive cases reported by de Leval, *et al* may represent "pattern 1" cases of AITL, without significant FDC proliferation.

Due to its restricted expression in lymphoid neoplasms, CD10 has been extensively used in the diagnosis of precursor B-and T-cell neoplasms and mature B-cell neoplasms, especially follicular lymphoma (Arber & Weiss, 1997;Chu & Arber, 2000;Dogan *et al*, 2000).

CD10 /NEP belongs to a family of membrane peptidases that includes, among others, structurally related leukocyte-associated molecules: CD26 and CD13 (Shipp & Look, 1993). CD10 is a monomeric 749-amino acid type II integral membrane peptide (Shipp *et al*, 1988). It contains a hydrophobic 24-amino acid transmembrane region that also functions as a signal peptide. The extracellular region contains a pentapeptide sequence that is associated with zinc binding and catalytic activity on cell surface, and zinc

dependant metalloproteases. CD10 is phosphorylated by casein kinase II, a serine and threonine kinase that increases in activity following signaling (Shipp *et al*, 1988;Shipp & Look, 1993). Cell surface CD10 acts to reduce cellular response to peptide hormones by regulating local peptide concentrations (Shipp *et al*, 1990;Shipp & Look, 1993). It hydrolyses peptide bonds on the amino side of the hydrophobic amino acids, valine, phenyl-alanine, isoleucine or tyrosine thereby reducing the local peptide concentration available for receptor binding and signal transduction (Shipp & Look, 1993). CD10 hydrolyses many substances such as endothelins, enkephalin, angiotensin, atrial natriuretic factor and bombesin among others (Shipp & Look, 1993). In systems such as lung (Shipp *et al*, 1988) and prostate cancer (Papandreou *et al*, 1998), CD10 has been shown to regulate tumour survival in vivo by decreasing extracellular neuropeptide concentrations and inhibiting certain signal transduction pathways (Sumitomo *et al*, 2000;Sumitomo *et al*, 2001). Although several non-haematopoeitic tissues were known to express NEP, its presence on lymphoid cells was not established until CD10 and NEP proved to be identical (Letarte *et al*, 1988). In haematopathology, CD10 was used as a diagnostic marker long before its function was known. In a study by Guerin *et al,* they show that treatment of fetal thymic organ cultures with specific CD10 inhibitors results in the inhibition of thymocyte differentiation, indicating that CD10 plays a specific role in promoting early T-cell development (Guerin *et al*, 1997). The T-cell leukaemia cell line Jurkat that expresses low levels of CD10/NEP has been used to show that CD10/NEP may function to regulate IL-2 production in certain T-cells. There are also in vivo and in vitro studies suggesting that CD10/NEP regulates B-cell development either by inactivating a peptide that stimulates B-cell proliferation and differentiation or by activating a pro-peptide that inhibits B-cell proliferation and differentiation (Salles *et al*,

1992). Despite its value as a diagnostic marker in B-cell lymphomas, there is very scant data on its physiological role in follicle center B-lymphocytes. This lack of information is surprising since most follicle center derived B-cell lymphomas express CD10 and there are proprietary pharmacological inhibitors of CD10 function (Weber, 2001).

3.3.3 Apoptosis and CD10 expression

In normal lymphoid tissues, the CD10 expression is largely restricted to B-cell compartments with typically high rate of apoptosis (Dogan *et al*, 2000). Of the B-cell neoplasms that express CD10, follicular lymphoma is associated with inhibition of apoptosis (Johnson *et al*, 1993) while Burkitt lymphoma is associated with a high rate of apoptosis (Blum *et al*, 2004;Mcgrath *et al*, 2001). In a recent study it was also shown that CD10 positive acute lymphoblastic leukaemia cells were in cell cycle with elevated c-myc levels and a propensity to apoptosis (Cutrona *et al*, 2002). CD10 expression is also induced in T-cells going through apoptosis (Bladon & Taylor, 2000;Cutrona *et al*, 1999). Using in vitro and in vivo experiments, Cutrona *et al* have shown that both CD4 positive and CD8 positive subsets of post thymic T-cells that do not normally express CD10, become CD10 positive when they undergo apoptosis induced by HIV infection and exposure to CD95 monoclonal antibody (Cutrona *et al*, 1999). This was only seen by flow-cytometric analysis but not by immunohistochemistry suggesting that the protein levels may be too low for detection on tissue sections. This close association with CD10 expression and apoptosis suggests that CD10 plays a role in regulation of lymphocyte survival. In fact, the relationship between CD10 and apoptosis was recently demonstrated in HL-60 human promyelocytic leukaemia cell line, where CD10/NEP was induced by exposure to a pro-apoptotic agent jaspakinolide (Cioca & Kitano, 2002).

Interestingly, CD10/NEP expression in turn, seemed to interfere with the apoptotic activity of the agent, because inhibition of CD10, enhanced apoptosis. Also in this study, CD10 expression was partially blocked by a broad-spectrum caspase inhibitor, further supporting the association of CD10 and apoptosis.

3.3.4 CD10 expression and regulation of apoptosis in AITL

It is possible that aberrant CD10 expression in neoplastic T-cells in AITL may be an indicator of disturbed apoptotic cell death. Previous studies (Feller, Namikawa, Willenbrock, Lee 2003) showed that the proliferating cells were CD4 positive, even in cases where CD8 positive cells predominated (Lee, 2003). Our findings showed that despite a high proliferation fraction, the CD10 positive tumour cells, which are greatly outnumbered by reactive lymphoid cells and account for only a proportion of the CD4 positive lymphoid cells, have a low proliferation fraction. This is analogous to low grade B-cell lymphomas such as the follicular lymphoma where prevention of cell death by over-expression of anti-apoptotic BCL2 protein rather than acquisition of high proliferative activity is considered to be the critical molecular event (Harris & Ferry, 2001). An attractive hypothesis is, analogous to follicular lymphoma, AITL is a biologically indolent/low grade tumour causing and its development and progression are driven by immune deregulation rather than increased tumour load. This is supported by the clinical observations suggesting that some patients do respond to immunosuppressive treatment (Advani, *et al* 1997; Murayama, *et al* 1992; Takemori, *et al* 1999). Elevated serum levels of Fas/CD95 were detected in AITL, but not in other NHL (Yufu *et al*, 1998). Following the publication of the results included in this thesis (Attygalle *et al*, 2002), it has been shown that the FDCs and endothelial cells in AITL

express Fas ligand (FasL) whereas the CD10 expressing tumour T-cells express Fas (CD95) and caspase 3, indicating Fas-FasL interaction between the neoplastic T-cell and the FDC and suggest that the follicular milieu is necessary for CD10 expression by tumour cells, a feature that may play a functional role in regulating apoptosis (Kim *et al*, 2002a).

3.3.5 Concluding remarks

The results of this part of the study show that CD10 is a marker of neoplastic T-cells in AITL. Identification of a specific marker for the neoplastic T-cells in AITL, for the first time, gives us the opportunity to investigate the biology of this disease with a view to devise novel therapeutic approaches.

Chapter 4

ANGIOIMMUNOBLASTIC T-CELL LYMPHOMA VERSUS PERIPHERAL T-CELL LYMPHOMA, UNSPECIFIED:

DISTINGUISHING HISTOLOGICAL, IMMUNOPHENOTYPIC AND MOLECULAR GENETIC FEATURES

4.1 Introduction

Diagnosis and classification of PTL in lymph nodes is challenging and important as different subtypes have different clinical outcomes and may require different treatment strategies. Nodal PTLs fall into the three broad categories of AITL, PTLu and ALCL. Diagnosis of ALCL is relatively easy and beyond the scope of this study. Distinguishing between PTLu and AITL can be much more difficult and often rests on subtle morphological and phenotypical differences. As was briefly mentioned in chapter 1, section 1.9.3.7, typical cases of AITL are characterised histologically, by a polymorphic infiltrate with or without clusters of clear cells, a prominent arborising vascular network of HEV, and more specifically by the FDC proliferation which shows a tendency to surround HEV (Jaffe & Ralfkiaer, 2001a). However, distinction from reactive conditions and PTLu, becomes quite difficult in cases with hyperplastic GCs ("pattern I histology" -described in chapter 3) and minimal FDC expansion and in cases with otherwise typical ("patterns II-III") morphology that lack significant FDC proliferation. The evidence in support of those with "pattern I" histology being AITL, comes mainly from the report by Ree and co-workers (1998), where 2 cases with hyperplastic GCs and molecular genetic

evidence of a monoclonal T-cell population progressed to more typical AITL on subsequent biopsy. This would also be consistent with the observations in the previous chapter (chapter 3), where the 3 overlapping histologic patterns (I-III) seemed to be consistent with a biologic continuum showing a progressive increase CD10+ neoplastic T-cells from patterns I-III. Nevertheless, there appears to be a considerable overlap between the morphology of less typical examples of AITL and the heterogeneous group categorised as PTLu.

The aims in this study are two-fold. The first aim was to compare the histology and molecular genetic profiles of AITL and PTLu.

The second aim was to assess the sensitivity and specificity of CD10 expression to AITL.

4.2 Results

4.2.1 Tissues used and categorization of subtypes

138 lymph node biopsies (from 138 patients) classified as PTL were retrieved from departmental archives and the histology and available immunohistochemistry reviewed and the diagnosis confirmed or revised in each case. The minimum immunohistochemistry available for review included CD3, CD20 and in most cases, CD4 CD8, and Ki67. In the cases of ALCL, CD30, and ALK-1 were also examined. In order to assess the FDC meshwork, immunohistochemistry was performed for CD21 in all cases of AITL and PTLu, and CD23 and CD35 in a limited number of cases. Categorisation of cases were as follows: AITL (n=89), PTLu (n=22), ALCL (n=16; 12 ALK-1 positive and 4 ALK-negative) and nodal involvement by MF (n=1). The cases of AITL were in turn subcategorized into those with pattern I (n=15), pattern II (n=17) and

pattern III (n=57) according to the presence of hyperplastic follicles, regressed follicles or absence of identifiable follicles (refer chapter 3). Of the cases categorised as pattern I, the original diagnoses made at our institution were as follows: AITL (n=10); reactive, but suspicious for PTL (n=1); PTL, T-zone variant (n=1); PTLu (n=3). In addition to these cases of PTL there were 10 cases that showed the typical morphology of AITL (Figure 1E) with effaced lymph node architecture by a polymorphous infiltrate and no identifiable residual follicular structures (pattern III histology described in chapter 3), a rich arborising vascular network, but only subtle expansion of the FDC meshwork with a tendency to surround vessels (Figure 1F). These cases were classified as "AITL/PTL-indeterminate", for the purposes of the study. The original diagnoses for these 10 cases included PTLu (n=6) and AITL (n=4).

4.2.2 Comparison of AITL, AITL/PTL - indeterminate and PTLu

4.2.2.1 Histology

In AITL, "AITL/PTL-indeterminate", and PTLu, the following features were evaluated and compared. On histology, the features examined included, the nature of the infiltrate (polymorphic vs monomorphic), degree of vascularity (mild, moderate, prominent) and the presence or absence of clusters of clear cells, the results of which are given in Tables 4.1 and 4.2. Of the 22 cases of PTLu reviewed, 2 cases showed an interfollicular pattern of growth (Figure 1P) while 3 cases showed features of the Lennert or lymphoepithelioid cell variant (Figure 1O). Likewise in AITL, the 15 cases categorized as pattern I showed an interfollicular "T-zone" pattern (Figure 1C), while 3 cases with pattern III histology showed a prominence of epithelioid cell histiocytes (Figure 1M)). The latter was also a feature in one of the cases categorized as AITL/PTL-indeterminate (Figure 1N). All

cases of AITL and AITL/PTL-indeterminate showed a prominent arborising vascular network, while the cases of PTLu showed a "mild" and occasionally "moderate" degree of vascularity. Clear cells were a feature in 41/89 (46%) cases of AITL, being more frequent in those with pattern III morphology (60%). They were a feature in 7/10 (70%) cases of AITL/PTL-indeterminate, but were not identified in any of the cases classified as PTLu.

Table 4.1. AITL and AITL/PTL-indeterminate: Histology and FDC evaluation

		Histology			Immunohistochemistry
Subtype (Number of biopsies	**Pattern (Number of biopsies)**	**Infiltrate/ Pattern**	**Vascularity**	**Number of biopsies with clear cells/total**	**CD21+ FDC meshwork**
AITL (n=90)	I (n=15)	Polymorphic/ Interfollicular	Prominent	1/15 (7%)	Follicles +/- subtle expansion
	II (n=17)	Polymorphic	Prominent	6/17 (35%)	Expanded meshworks surrounding vessels
	III (n=57)	Polymorphic	Prominent	34/57 (60%)	Expanded meshworks surrounding vessels
AITL/PTL-indeterminate (n=10)	III	Polymorphic	Prominent	7/10 (70%)	Subtle expansion within close vicinity of and surrounding vessels

Abbreviations: AITL, angioimmunoblastic T-cell lymphoma; PTL, peripheral T-cell lymphoma; +, positive; FDC, follicular dendritic cell.

4.2.2.2 Immunohistochemistry

4.2.2.2.1 *Evaluation of the FDC meshwork*

The FDC meshwork in all 3 categories was evaluated using immunohistochemistry (Tables 4.1 and 4.2). In AITL, patterns II and III histology, the FDC meshwork showed characteristic proliferation with a tendency to surround HEV (Figure 1B). In the cases categorised as "pattern I" the FDCs was more or less confined to the follicles with subtle expansion in some of the follicles (Figure 1D). In 4 of the 10 cases categorized as

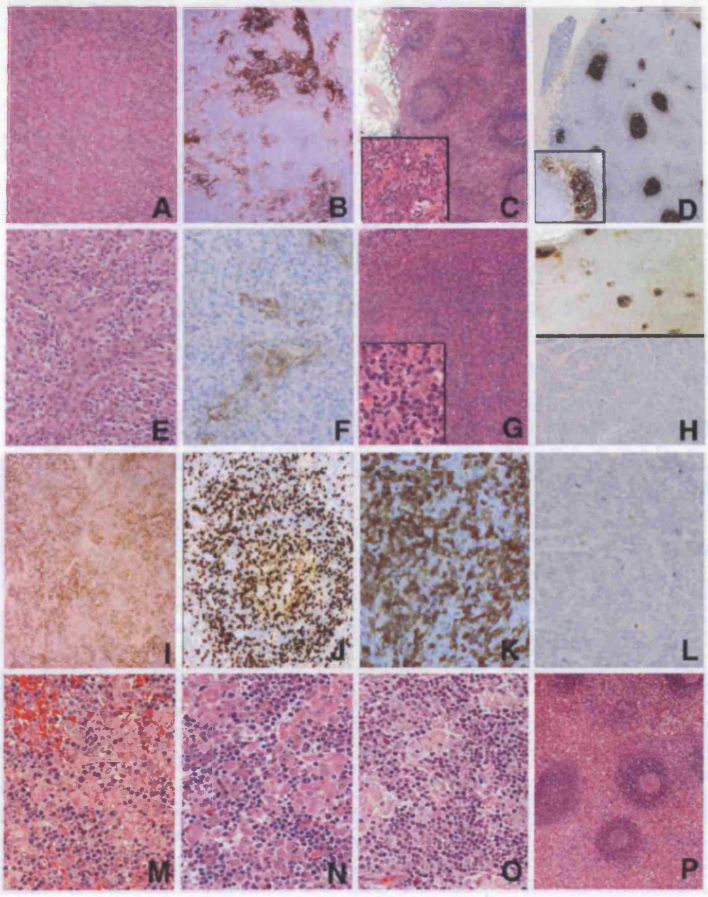

Figure 4.1 Morphology, follicular dendritic cell (FDC) meshwork and CD10 expression in AITL, AITL/PTL indeterminate and PTLu.

Panels A and B show an example of AITL with typical morphology. (A) Haematoxylin and eosin (H&E) stained section of a typical ("Pattern III") AITL. (B) CD21 highlights the marked expansion of FDC.
Panels C and D show "Pattern I" AITL. (C) H&E stained section of "Pattern I" AITL showing hyperplastic follicles. The inset shows a high power view of the paracortex with a prominence of vessels. (D) CD21 shows subtle (inset)/no significant expansion of FDC.
Panels E and F show an example of "AITL/PTL indeterminate". (E) H&E stained section shows the typical morphology of AITL with a rich vascular network and a polymorphous infiltrate. (F) CD21 shows subtle expansion of FDC with a tendency to encircle vessels.
Panels G and H show a case of PTLu. (G) H&E stained section of PTLu showing a monomorphic infiltrate of atypical lymphoid cells. The inset shows a high power view of the same. (H) CD21 staining shows an example of PTLu with residual follicles (H, upper panel) and an example with no residual follicles (H, lower panel).
(I, J, K and L) CD10 immunostaining shows numerous CD10 positive lymphoid cells in a case of typical AITL (I), "Pattern I" AITL (J) and AITL/PTL indeterminate (K), and absence of CD10 positivity in PTLu (L).
Panels M-O show examples of nodal PTL with a lympho-epithelioid/Lennert morphology. (M) H&E stained section of a case of AITL. (N) H&E stained section of a case of AITL/PTL indeterminate. (O) H&E stained section of a case of PTLu.
Panel P shows an H&E stained section of a case of PTLu that showed a monomorphic infiltrate in a interfollicular distribution.

AITL/PTL-indeterminate (see above), CD23 and CD35 expression was also evaluated, but showed no expansion of FDCs in excess of the subtle increase observed with CD21. In the 22 cases of PTLu, the FDC was either absent or present in the form of residual or compressed follicles (Figure 1H).

Table 4.2. PTLu: histology and FDC evaluation by immunohistochemistry

Case no	Histology			Immunohistochemistry
	Infiltrate/ Pattern	**Vascularity**	**Clear cells**	**CD21+ FDC meshwork**
1	Polymorphic	Mild	Absent	Follicles
2	Polymorphic	Mild	Absent	Follicles
3	Polymorpphic (LEL)	Mild	Absent	Compressed follicles
4	Monomorphic	Mild	Absent	Compressed follicles
5	Polymorphic	Mild	Present	Compressed follicles
6	Monomorphic	Mild	Absent	Follicles
7	Monomorphic	Mild	Absent	Compressed follicles
8	Monomorphic/ interfollicular	Mild	Absent	Follicles
9	Polymorphic	Mild	Absent	Compressed follicles
10	Polymorphic	Mild	Absent	Follicles
11	Polymorphic	Mild	Absent	Absent
12	Polymorphic	Mild	Absent	Follicles
13	Polymorphic	Mild	Absent	Compressed follicles
14	Monomorphic	Mild	Absent	Follicles
15	Polymorphic	Mild	Absent	Follicles
16	Polymorphic (LEL)	Mild	Absent	Follicles
17	Polymorphic (LEL)	Mild	Absent	Absent
18	Polymorphic/ Interfolliclular	Mild	Absent	Follicles
19	Monomorphic	Mild	Absent	Follicles
20	Polymorphic/ Interfollicular	Mild	Absent	Follicles
21	Polymorphic	Mild	Absent	Follicles
22	Monomorphic	Moderate	Absent	Compressed follicles

Abbreviations: +, positive; FDC, follicular dendritic cell; LEL, lymphoepithelioid morphology.

4.2.2.3 Molecular genetics

4.2.2.3.1 *EBER-ISH and immunoglobulin heavy chain (IgH) gene PCR*

EBER-ISH was used to assess for the presence of EBV-infected lymphoid cells. If only an occasional EBER-positive cell could be identified, this was interpreted as negative for expansion of EBV-infected cells. The results are summarized in Figure 4.2 and Table 4.3, and show that expansion of EBV infected B-cells was observed in AITL (81%) and AITL/PTL-indeterminate (57%) but not in PTLu. PCR for IgH gene rearrangement (Table 4.3) showed a monoclonal/oligoclonal pattern (1-2 dominant bands/3-4 dominant

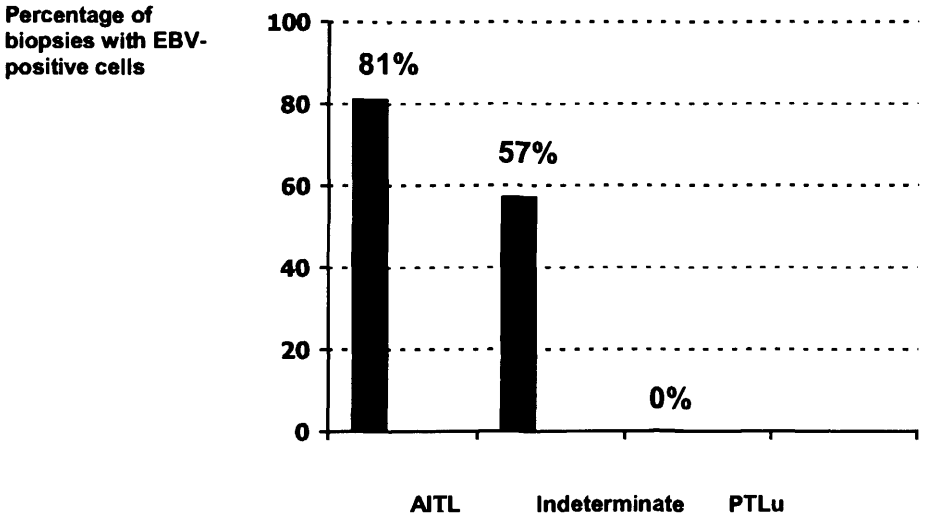

Figure 4.2 Percentage of biopsies with EBV-positive cells

Abbreviations: AITL, angioimmunoblastic T-cell lymphoma; Indeterminate, AITL/PTL indeterminate; PTLu, peripheral T-cell lymphoma, unspecified; EBER+, Epstein Barr virus encoded RNA positive

bands) in 19% of AITL but no dominant band/bands were identified in the cases of AITL/PTL-indeterminate or PTLu.

Table 4.3. AITL, AITL/PTL-indeterminate and PTLu: EBER-ISH and PCR for IgH gene re-arrangement.

Nodal PTL subtype	Pattern	Number of EBER+ biopsies/ #Total (%)		PCR-IgH Mono-oligoclonal/ #Total (%)	
AITL	I	7/10 (70%)	47/58 (81%)	0/15	14/73 (19%)
	II	11/14 (85%)		2/15 (13%)	
	III	27/34 (80%)		12/43 (28%)	
AITL/PTL-indeterminate	III	4/7 (57%)		0/9	
PTL unspecified		0/10		0/22	

Abbreviations: PTL, peripheral T-cell lymphoma; AITL, angioimmunoblastic T-cell lymphoma; EBER, Epstein Barr virus encoded RNA; +, positive; PCR, polymerase chain reaction; IgH, immunoglobulin heavy chain gene
Total number of cases that showed a conclusive result

4.2.2.3.2 PCR for T-cell receptor γ (TCRγ) gene rearrangement

The PCR results are given in Figure 4.3 and Table 4.4. The presence of 1-2 dominant bands and 3-4 dominant bands was interpreted as a monoclonal and oligoclonal result respectively. A monoclonal or oligoclonal result was obtained in 90% of the cases of AITL, 88% AITL/PTL-indeterminate and 59% of the cases of PTLu. This difference (between AITL and PTLu) was statistically significant (p=0.0006). There was no significant difference in the proportion of cases with an oligoclonal result.

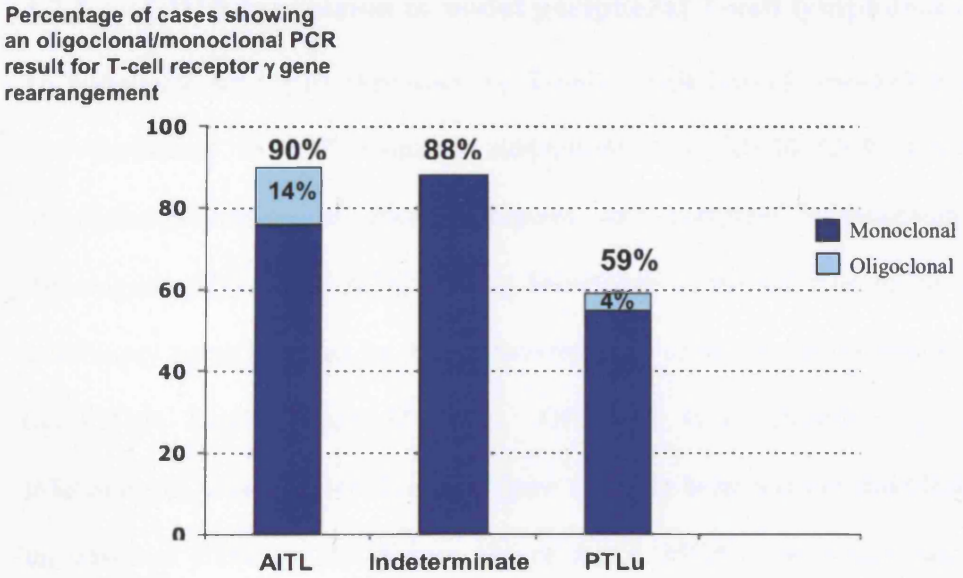

Figure 4.3 Percentage of cases showing an oligoclonal/monoclonal PCR result for T-cell receptor γ gene rearrangement.

Abbreviations: AITL, angioimmunoblastic T-cell lymphoma; Indeterminate, "AITL/PTL indeterminate; PTLu, peripheral T-cell lymphoma, unspecified

Table 4.4: PCR for T-cell receptor γ gene rearrangement

Abbreviations: PTL, peripheral T-cell lymphoma; AITL, angioimmunoblastic T-cell lymphoma; PCR,

Nodal PTL subtype	Pattern	TCRγ gene PCR: mono-clonal/ #Total (%)		TCRγ oligo-clonal/#Total (%)		PCR-TCRγ gene PCR: mono-oligoclonal/ Total# (%)	
AITL	I	11/14 (79%)	66/87 (76%)	3/14 (21%)	12/87 (14%)	14/14 (100%)	78/87 (90%)
	II	14/16 (88%)		0/16		14/16 (88%)	
	III	41/57 (72%)		9/57 (16%)		50/57 (88%)	
AITL/PTL-intermediate	III	7/8 (88%)		0/8		7/8 (88%)	
PTL unspecified		12/22 (55%)		1/22 (4%)		13/22 (59%)	

polymerase chain reaction; TCRγ gene
Total number of cases that showed a conclusive result

4.2.3 CD10 expression in nodal peripheral T-cell lymphomas

To investigate for CD10 expression by T-cells, single-layered immunohistochemistry was performed in 137 biopsies, supplemented by CD20/CD10 double-layered immunohistochemistry in selected biopsies, and evaluated independently by two Pathologists (A.D.A and A.D), without knowledge of the sub-type of the individual case/biopsy being assessed i.e. blind. Seventy-eight of the 88 (89%) biopsies of AITL had CD10+ T-cells (Figure 1I and J). Of the 10 cases categorized as AITL/PTL-indeterminate, 6 had CD10+ T-cells (Figure 1K). The latter was not identified in any of the cases of PTLu (0/22) (Figure 1L) or ALCL (0/16). The single case of nodal involvement by MF contained many CD10+ T-cells, consistent with the neoplastic population. In addition to the cases mentioned, 10 reactive lymph nodes were also double-stained with CD20/CD10, but no CD10+ T-cells were identified.

Table 4.5 CD10 expression in nodal peripheral T-cell lymphomas

Nodal PTL subtype	Pattern	Number of biopsies with CD10+ T-cells / Total (%)	
AITL	I	15/15 (100%)	78/88 (89%)
	II	15/17 (88%)	
	III	48/56 (86%)	
AITL/PTL- indeterminate	III	6/10 (60%)	
PTL unspecified		0/22	
ALCL		0/16	
Secondary node involvement by mycosis fungoides		1/1 (100%)	

Abbreviations: PTL, peripheral T-cell lymphoma; AITL, angioimmunoblastic T-cell lymphoma; ALCL, anaplastic large cell lymphoma; +, positive

4.3 Discussion

4.3.1 Frequency of AITL

PTLu includes a heterogenous group of yet undefined entities, whereas AITL is believed to represent a distinct clinico-pathological entity that accounts for approximately 15-20% of all nodal PTLs (1997). Although most cases in this study are from a referral practice and thus probably not entirely representative, AITL (patterns I, II and III) seems much more frequent than is reported in the literature, and accounts for approximately 65% of nodal PTLs reviewed.

4.3.2 Histologic features of AITL versus PTLu

Clear cells, once considered to be an important feature of AITL (Nathwani & Jaffe, 1995), are no longer regarded as essential for diagnosis (Jaffe & Ralfkiaer, 2001a). In the present study, clear cells were observed in <50% of cases of AITL. They were present in the 7 of 10 cases of AITL/PTL-indeterminate but were not observed in any cases categorised as PTLu. Although the assessment in this study was subjective, the rich arborising-vascular network observed in cases of AITL and AITL/PTL-indeterminate was not seen in any of the cases of PTLu.

4.3.3 AITL with hyperplastic follicles (pattern 1) versus T- zone variant of PTLu

Diagnosis of cases of AITL that fulfill the defined criteria laid down by the WHO (Jaffe & Ralfkiaer, 2001a) is fairly straightforward. Although for many years, the presence of

hyperplastic follicles was thought to exclude a diagnosis of AITL, the cases described by Ree and co-workers (1998) showing hyperplastic follicles, have been included as AITL in the WHO classification (Jaffe & Ralfkiaer, 2001a). It is believed that such cases are rare and may show progression to typical AITL (Jaffe & Ralfkiaer, 2001a). In the current study these cases (pattern I) account for 15/89 (17%) cases of AITL, suggesting that the "rarity" reported, may in part be due to difficulty in distinguishing it from reactive conditions and the T-zone variant of PTLu. It is in fact impossible to distinguish "T-zone" PTLu - a histologic variant that is described as having an interfollicular growth pattern with preserved or hyperplastic follicles, a polymorphous infiltrate +/- clusters of clear cells, scattered RS-like cells and prominent HEV (Ralfkiaer *et al*, 2001) - from AITL "pattern I", and calls to question whether they are in fact the same entity – i.e. early AITL. Clinically there is a clear overlap between AITL and T-zone variant of PTLu, formerly known as T-zone lymphoma (Helbron *et al*, 1979) and at a cytogenetic level, trisomy 3, the most frequent abnormality described in AITL, has also been reported in "T-zone lymphoma" (Schlegelberger *et al*, 1994a). In the current study, CD10+ T-cells were present in all 15 cases with "pattern I" histology, a phenomenon that was observed in almost 90% of typical AITL, but not in a single case categorized as PTLu, again supporting the view that many of these fall within the spectrum of AITL.

4.3.4 AITL with a prominence of epithelioid cells versus lympho-epithelioid variant of PTLu

Lymphoepithelioid or Lennert lymphoma was initially described by Karl Lennert (1968) prior to the concept of immunophenotyping and thus included a heterogeneous

group of entities characterised by a prominence of epithelioid histiocytes (Kim *et al*, 1980). It is now regarded as a histological variant of PTLu, and not as a specific subtype. In the current study the presence of numerous clusters of epithelioid hisiocytes was noted in 3 cases of AITL, in 1 case categorised as AITL/PTL-indeterminate and in 3 cases of PTLu, confirming the view that this histologic appearance is not specific to any given subtype (Kim *et al*, 1980;Patsouris *et al*, 1990). Gisselbrecht and co-workers (Gisselbrecht *et al*, 1998) show that there is an overlap in survival curves in patients with AITL and the lymphoepithelioid variant of PTLu and comment on the difficulty in distinguishing the two entities morphologically, a problem that is also reported by Nakamura and Suchi (Nakamura & Suchi, 1991). The overlap is also reported at a genetic level, with the frequent occurrence of trisomy 3 in both entities (Schlegelberger *et al*, 1994a).

4.3.5 EBV infection and dominant B-cell clones in AITL and PTLu

Although secondary EBV infection has been described in PTLu (Quintanilla-Martinez *et al*, 1999;Zettl *et al*, 2002), none of the PTLu in the present study showed evidence of expansion of EBV-infected lymphoid cells. However, 81% of AITL and 67% of cases of AITL/PTL-indeterminate showed EBV-infected lymphoid cell. This frequency is not as high as that documented in some studies that report EBV-positivity in over 95% of the cases of AITL (Anagnostopoulos *et al*, 1992;Weiss *et al*, 1992). The presence/absence of EBV infected B-cells in the present study correlates well with the outgrowth of dominant B-cell clones in 19% of AITL and its absence in PTLu.

4.3.6 Detection of a dominant T-cell clone: AITL versus PTLu

T-cell clonality analysis enabled detection of a monoclonal or oligo-clonal T-cell population in approximately 90% of AITL whereas this was possible in approximately 59% of PTLu. Although the reason for this difference remains unknown, it may reflect the presence of multiple dominant T-cell clones /oligo-clones in AITL, at least one of which would bind the primers used for clonality analysis and give rise to a dominant band. There was however no statistically significant difference in the frequency of an oligoclonal result between the two groups. The identification of a monoclonal T-cell population in all 14 "pattern I" cases that had a conclusive PCR result may reflect the fact distinction from reactive lymphoid hyperplasia and a definite diagnosis of T-cell lymphoma in these cases is often difficult without the support of a T-cell clonal molecular genetics result. Although at the time of performing this work the BIOMED-2 protocols (van Dongen *et al*, 2003) were not published, it would be useful to evaluate these cases using the BIOMED-2 TCRγ, and (if the DNA quality is satisfactory) TCRβ primer sets, to validate these results.

4.3.7 AITL/PTL- indeterminate or AITL?

The 10 cases categorised as AITL/PTL indeterminate showed morphologic similarity to AITL with a polymorphous infiltrate and pronounced vascularity. Clusters of clear cells, a characteristic, but not universal feature of AITL were also present in 7/10 cases. Although they lacked the florid FDC proliferation expected with "pattern III" histology, the FDCs present did show a tendency to surround vessels, yet another feature of AITL.

As in AITL, the expansion of EBV-infected B-cells was a feature in most cases. However, despite the evidence of expanded EBV infected B-cells, there was no evidence of outgrowth of a dominant B-cell clone in these cases. The pattern of T-cell clonality in these cases was similar to AITL, with a dominant clone/clones being identified in a very high proportion (88%) of cases. CD10+ T-cells were also a feature in the 6/10 of cases, a feature not observed in PTLu. Overall the features of AITL/PTL-indeterminate suggest that they fall within the spectrum of AITL, rather than within the heterogeneous group of PTLu. The lower percentage of CD10 expression in these cases maybe due to the lack of the expected degree of FDC proliferation seen in more typical cases with similar "pattern III" morphology.

4.3.8 CD10 expression in nodal T-cell lymphomas

CD10 positive T-cells were observed in 86% of typical AITL (patterns II and III) but not in PTLu or ALCL. This phenomenon seems to be a sensitive marker of AITL. The specifity would depend on how the cases classified as AITL, pattern I histology and AITL/PTL-indeterminate are categorised. There is some recent evidence (Ree *et al*, 1998) that suggests that those cases with pattern I histology represent early AITL, and the evidence in this part of the study suggests that the AITL/PTL-indeterminate cases fall within the spectrum of AITL. If so, in the assessment of nodal PTLs, CD10 expression is not only approximately 85% sensitive but also 100% specific to AITL. CD10 expression by the neoplastic cells in secondary nodal involvement by MF was an unexpected finding. Although there is a single report of CD10 expression in SS (Chubachi *et al*, 1994), this is not a recognized feature of MF. Nevertheless, as the clinical scenario and histology is distinct from AITL, CD10 expression in this case

should not lead to a mistaken diagnosis of AITL. The only mention of CD10 expression in nodal PTLs in the literature is the report by de Leval, *et al* (2001) describing a nodal PTL with a follicular growth pattern. These cases probably represent early AITL, rather than a distinct subtype of PTL.

Although Cook and co-workers (2003) report the occurrence of a small subset of reactive CD10+ T-cells in some reactive lymph nodes and B-cell lymphomas, we did not identify any CD10+ lymphoid cells that could be interpreted as presumptive T-cells, in reactive lymph nodes. In their study Cook and co-workers use flow-cytometry and PAX-5/CD10 double-layered immunohistochemistry. The combination of a nuclear stain (PAX-5) and a membrane stain (CD10) may be superior to the method used in our study i.e. two membrane stains (CD20 and CD10), when trying to identify small numbers of CD10+ T-cells.

4.3.9 Is AITL underdiagnosed?

If the cases of AITL/PTL-indeterminate are included as AITL, the latter accounts for 72% of all nodal PTL reviewed, a figure far in excess of that reported in the literature. Although as mentioned previously this may represent a sample bias, it may partly be due to the fact that many cases of AITL that do not show "typical" morphology are probably categorised as PTLu or misdiagnosed as reactive. It is interesting to note that a case similar to that described by Macon and co-workers (Macon *et al*, 1995) as "paracortical nodular T-cell lymphoma", believed at the time to be an unusual variant PTL, has recently (2004) been diagnosed as AITL by WR Macon and A Dogan (Figure 4.4 - personal communication). This example shows "pattern II" morphology, with, regressed follicles, increased vascularity and clusters of clear cells (T-cells), and scattered RS-like

B- cells amidst a polymorphic background (Figure 4.4). There is immunohistochemical evidence of FDC expansion, but the T-cells are CD10 negative. Rudiger and co-workers (Rudiger *et al*, 2000) describe 9 cases of PTL with a distinct perifollicular growth pattern showing a prominence of HEV, regressed follicles and clusters of cells with pale to clear cytoplasm in a marginal zone distribution. In some of these cases, a close association between the HEV and clear cells was noted and in the majority, scattered Hodgkin-like cells were noted in the background. EBV-infected by-stander B-cells were however noted in only 1/8 cases. Dense FDC meshworks identified by CD23 staining, was present in the follicle centers of all 9 cases described. Although CD23 highlights only a subset of the FDCs, and there is no proof that CD21 or CD35 is superior in the assessment of FDC expansion in AITL (Bagdi *et al*, 2001), it may be useful to assess the FDC meshworks in these cases with CD21 and may be CD35. The question remains whether at least some of these cases represent AITL.

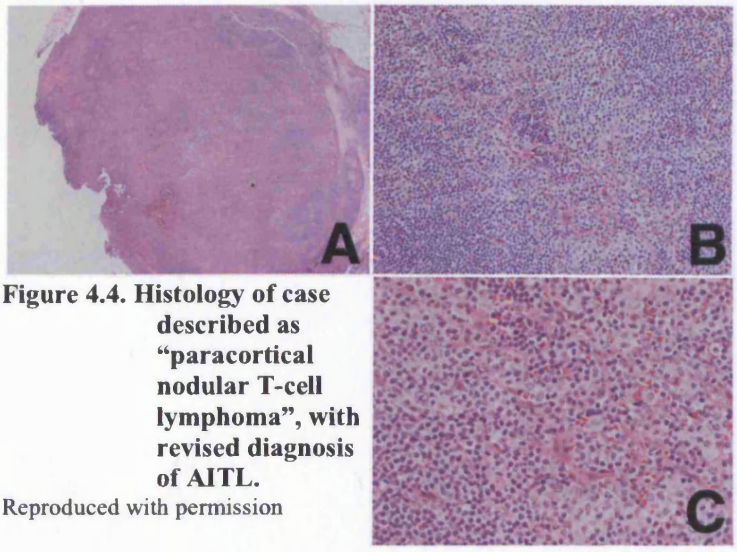

Figure 4.4. Histology of case described as "paracortical nodular T-cell lymphoma", with revised diagnosis of AITL.
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4.3.10 Concluding remarks

In conclusion, CD10 expression seems to be a sensitive and specific marker of the disease and a useful adjunct to the diagnosis of AITL. The findings in this study suggest that the diagnostic criteria for AITL need to be revised, as the existing pathological criteria apply only to the "typical" histology that probably accounts for approximately 80% of all cases.

Chapter 5

CD10 EXPRESSION IN EXTRANODAL DISSEMINATION OF ANGIOIMMUNOBLASTIC T-CELL LYMPHOMA

5.1 Introduction

AITL is a nodal PTL characterised by systemic disease and prominent constitutional symptoms (Frizzera *et al*, 1974;Siegert *et al*, 1992;Siegert *et al*, 1995). Although generalised lymphadenopathy is a prominent feature, clinical evidence of extranodal involvement is often present at diagnosis. These include hepato-splenomegaly seen in 50-70%, skin rash in 50%, pleuro-pulmonary involvement in 40% and bone marrow involvement in 60-80% of patients (Frizzera *et al*, 1974;Ghani & Krause, 1985;Pautier *et al*, 1999;Siegert *et al*, 1992;Siegert *et al*, 1995;Starke *et al*, 1983;Weisenburger *et al*, 1977). The almost universal occurrence of lymphadenopathy permits a diagnosis based on examination of a lymph node biopsy and extranodal sites, other than bone marrow are rarely subjected to histologic examination. However occasionally an extranodal site will be biopsied either as a diagnostic procedure or to examine the extent of tumour involvement and rule out infectious or inflammatory conditions. In these situations diagnosis can be very challenging as the conventional criteria based on alterations in lymph node biopsies are not applicable. Histologically, involved lymph nodes show partial or total obliteration of the normal architecture by a polymorphic infiltrate of lymphocytes, plasma cells and eosinophils, prominent proliferation of venules and expansion of the FDC meshwork (Frizzera, 2001;Nathwani & Jaffe, 1995). Collections

of cells with pale to clear cytoplasm, described as being typical for AITL is not a consistent finding and in many instances cytological features of malignancy are not readily identifiable (Jaffe & Ralfkiaer, 2001a). Therefore, despite histologic criteria, definite diagnosis is often difficult even on lymph node biopsy, leading to an error in initial diagnosis in over 50% of the cases (refer chapter 3), further complicating histologic interpretation of an extranodal biopsy.

In chapters 3 and 4, in the study of lymph nodes in AITL, we showed that the neoplastic T-cells in most cases can be identified by CD10 expression, a feature absent in other nodal PTLs. Early and accurate diagnosis of AITL in lymph nodes can thus be achieved by immunostaining for CD10. CD10 expression if maintained at extranodal sites of involvement would serve as a phenotypic marker and a very useful diagnostic tool. The aim of the present study was to investigate whether the expression of CD10 by neoplastic T-cells is maintained in extranodal sites.

5.2 Results

5.2.1 Tissues used in this study

Seventy-eight cases of AITL diagnosed on clinical, histologic, immunophenotypic and molecular genetic criteria on lymph node biopsy were retrieved from the archives of the Department of Histopathology, University College London Hospital. Of these, 10 (referral) cases included biopsies of involved extranodal sites and were selected for study.

5.2.2 Clinical features

The clinical presentations and the initial diagnoses at referral are summarised in Table 5.1. Among the 10 cases studied, there were 5 females and 5 males between the ages of 33 and 82 years. In 5 of the 7 cases where clinical history was available, systemic symptoms were prevalent at some time during the course of the disease. Six cases had involved bone marrow trephines, 2 had pulmonary involvement, 1 had tonsillar and caecal involvement while 2 cases had involved nasopharyngeal biopsies. Lymph node biopsies were performed for initial diagnosis in 9 of the 10 cases, while in 1 case (case 2) a lung biopsy was followed 2 years later by a lymph node biopsy. An initial diagnosis of AITL was made in only 5 of the 10 cases.

5.2.3 Histology

5.2.3.1 Lymph nodes

Lymph node biopsies in cases 1-8 and 10 conformed to that described as typical for AITL, with effacement of architecture by a polymorphic infiltrate comprising, small lymphocytes and transformed blasts, plasma cells, histiocytes and eosinophils within a prominent vascular network (Figure 5.3A). A proportion of the lymphoid blasts in cases 1,2 and 4 had pale to clear cytoplasm. Follicles were either not identifiable or regressed with concentrically arranged FDCs. The pattern of involvement in case 9, was similar to that described by Ree and co-workers (1998), with preserved hyperplastic follicles, and a paracortical infiltrate similar to that seen in the other cases.

Table 5. 1 Clinical features, site of biopsy and initial diagnosis

Case no	Age/sex	Clinical presentation	Site of biopsy	Initial diagnosis
1	33 F	Not available	Lymph node	AITL
			Tonsil	Not available
			Caecum (1 year after diagnosis)	Recurrent lymphoma or early inflammatory bowel disease
2	75F	Shortness of breath and generalised lymphadenopathy. Chest X-ray showed an expanding discrete lesion in the right upper lobe	Lung	Reactive lymphoid hyperplasia
			Inguinal lymph node (2 years after lung biopsy)	Hodgkin lymphoma
		2-years later she re-presented with malaise, weight loss, hepatosplenomegaly and lymphadenopathy	Bone marrow	Staging -?involved
3	82M	B-symptoms, anaemia, generalised lymphadenopathy, splenomegaly.	Lymph node	Reactive-?connective tissue disorder
			Bone marrow	Staging - ? involved
4	43F	Lymphadenopathy 2 years later, presented with pneumonitis	Lymph node	AITL
			Lung	? involved by AITL
5	48M	Not available	Lymph node	AITL
			Bone marrow	Staging - ?involved
6	63F	Presented with pulmonary embolism and subsequently shown to have splenomegaly and cervical and abdominal lymphadenopathy	Cervical lymph node	AITL or PTL
			Bone marrow	Staging - ? involved
7	67M	Bilateral axillary and inguinal lymphadenopathy	Inguinal lymph node	High grade NHL
			Bone marrow	Staging -?involved
8	58M	Generalised lymphadenopathy and skin rash	Axillary lymph node	AITL
			Bone marrow	Staging –involved
9	81F	Not available	Cervical lymph node	NHL
			Nasopharynx	NHL
10	59M	Large mass in post-nasal space and multiple enlarged cervical lymph nodes	Cervical lymph node	Lennert's lymphoma
			Nasopharynx	Lennert's lymphoma

Abbreviations: F, female; M, male; AITL, angioimmunoblastic T-cell lymphoma; PTL, peripheral T-cell lymphoma; NHL, non-Hodgkin's lymphoma.

5.2.3.2 Extranodal sites

The histologic features of extranodal sites of involvement of each case are summarised in Table 5.2. The features ranged from a non-specific mixed infiltrate (bone marrow biopsies in cases 5,6 and 7) to a polymorphic infiltrate with clear cells in close proximity to a prominent arborizing vascular network (tonsillar and caecal biopsies in case 1) (Figure 5.1 A, B and G). All 6 involved bone marrow trephines showed focal involvement (paratrabecular in 4 cases). Two cases showed an increase in vascularity (Figure 5.2G). Three involved trephines (cases 5,6, and 7) showed a mixed infiltrate of small and large lymphoid cells. One case (case 3) had evidence of regressed follicles in close association with the neoplastic infiltrate, while the 2 biopsies with increased vascularity (case 2 and 8) comprised foci with small and large lymphocytes, epithelioid histocytes, fibroblasts and eosinophils amidst the vessels imparting a "granulomatous" appearance (Figure 5.2G).

5.2.4 Immunohistochemistry

Single-layered immunohistochemistry for CD3, CD20, CD10 and CD21 was performed in all cases and double-layered immunohistochemistry using CD20/CD10 in selected cases. In order to demonstrate CD10 expression in neoplastic T-cells, CD10 and CD20/CD10 sections were compared with sequential CD3 immunostained sections.

5.2.4.1 Lymph nodes

The infiltrate comprised a predominance of CD3 positive T-cells (Figure 5.3C). In cases 1-8 and 10, CD 21 highlighted the hyperplastic FDC meshwork that extended into the paracortex to surround HEV (Figure 5.3B). In case 9, CD21 immunostaining was more

or less confined to the hyperplastic follicles identified on histology, with only subtle extensions into the paracortex. Single layered immunohistochemistry with CD10 and double staining with CD20/CD10 highlighted a population of CD10 positive, CD20 negative lymphoid cells in 8 cases (cases 3-10) (Figure 5.3D-F). Consistent with the results of chapter 3, examination of sequential sections immunostained for CD3 and CD20/CD10 showed that these CD10 positive cells were the neoplastic T-cells. The distribution of these cells was similar to that of the expanded FDC meshwork. In case 9 that had hyperplastic follicles, the CD10 positive T-cells were situated at the outer rim of the follicle spilling into the paracortex. No CD10 positive T-cells were identified in the lymph node biopsy in case 2. CD10 expression could not be investigated in the lymph node biopsy in case 1 as no spare material was available.

5.2.4.2 Extranodal sites

CD3 immunostaining highlighted a marked T-cell infiltrate in all cases (Figure 5.1 D and I, Figure 5.2D and I and Figure 5.2H). The details of CD21 expression and CD10 expressing T-cells are given in Table 5.2. At one end of the spectrum, CD21 immunostaining showed mild expansion of the FDC meshwork as in the tonsillar and caecal biopsies of case 1 (Figure 5.1C and H). Focally, the FDCs surrounded the venules in these biopsies. This feature was subtle but nevertheless present in the lung biopsy of case 2 (Figure 5.2C). At the other extreme there were no CD21 positive FDC meshworks in 5 of the 6 bone marrow biopsies (Figure 5.2H). In all other biopsies the FDC meshwork highlighted either hyperplastic or regressed follicles but showed no expansion (Figure 5.3G). CD10 immunostain and CD20/CD10 double immunostain highlighted CD10 positive, CD20 negative lymphoid cells corresponding to T-cells in all

sites except in the bone marrow (Figure 5.1E, F, J and K, and Figure 5.2E and F). In the bone marrow, the presence of non-neoplastic haematopoietic precursors and the subtle nature of involvement made assessment of CD10 positivity of the neoplastic cells a little more difficult (Figure 5.2K). Nevertheless after careful comparison with morphology and CD3 immunostain, CD10 expression by neoplastic T-cells, could be identified only in one involved bone marrow trephine (Figure 5.3I). CD10 expression in all involved extranodal sites correlated well with the presence and distribution of FDCs (Figures 5.2, 5.2 and 5.3). In those cases where the FDCs showed minimal expansion/sprouting (lung biopsies in cases 2 and 4, bone marrow biopsy in case 3 and nasopharyngeal biopsy in case 9), CD10 positive T-cells were seen at the edge of the follicle spilling into the adjacent interfollicular region (Figure 5.3I).

5.2.5 In situ hybridisation for EBV-EBER

EBER-ISH was performed on lymph nodes in 2 – 10. Lymph nodes in cases 2-8 and 10 showed hybridisation for EBV- EBER in a subset of cells. Case 9 was EBER-ISH negative. EBER-ISH performed on the caecum in case 1 and the nasopharyngeal biopsy in case 9 was negative.

5.2.6 PCR for TCRγ chain gene

The results of PCR for TCRγ chain gene are shown in table 5.2. All but one case with good quality DNA showed either a single or two distinct bands, consistent with monoclonal T-cell population. Case 1 showed 3 bands and was interpreted as being biclonal/oligoclonal.

Table 5.2 Summary of histology, immunophenotypic profile and T-cell clonality analysis of involved extranodal biopsies

Case	Extranodal	Histology			Immunohistochemistry		TCR PCR
no	site	Follicles	Vascularity	Clear cells	CD21+ FDC meshwork	CD10+ T-cells	
1	Tonsil	Occasional regressed	Rich network	Present	Expanded with sprouts encircling vessels	Numerous	Not done
	Caecum	No identifiable follicles	Rich network	Present	Expanded with sprouts encircling vessels	Numerous	Oligoclonal
2	Lung	Hyperplastic	Inter-follicular increase	Absent	Highlights follicles; one focus expanded with sprouts	Many	Monoclonal (same size band as lymph node)
	Bone marrow	No identifiable follicles	Increased	Absent	Absent	Absent	Monoclonal (same size band as lymph node and lung)
3	Bone marrow	Regressed follicles	No increase	Absent	Highlights regressed follicles	Present	Poor DNA
4	Lung	No identifiable follicles	Increased	Present	Highlights follicles; no sprouts	Present	Monoclonal (same size band as lymph node)
5	Bone marrow	No identifiable follicles	No increase	Absent	Absent	Absent	Poor DNA
6	Bone marrow	No identifiable follicles	No increase	Absent	Absent	Absent	Monoclonal (same size band as lymph node)
7	Bone marrow	No identifiable follicles	No increase	Absent	Absent	Absent	Monoclonal (same size band as lymph node)
8	Bone marrow	No identifiable follicles	Increased	Absent	Absent	Absent	Not done
9	Nasopharyn-geal biopsy	Hyperplastic	Inter-follicular increase	Absent	Highlights follicles	Present	Inconclusive
10	Nasopharyn-geal biopsy	No identifiable follicles	Increased	Absent	Expanded with sprouts encircling vessels	Present	Monoclonal

Abbreviations: FDC, follicular dendritic cells; TCR, T-cell receptor γ chain gene; PCR, polymerase chain reaction

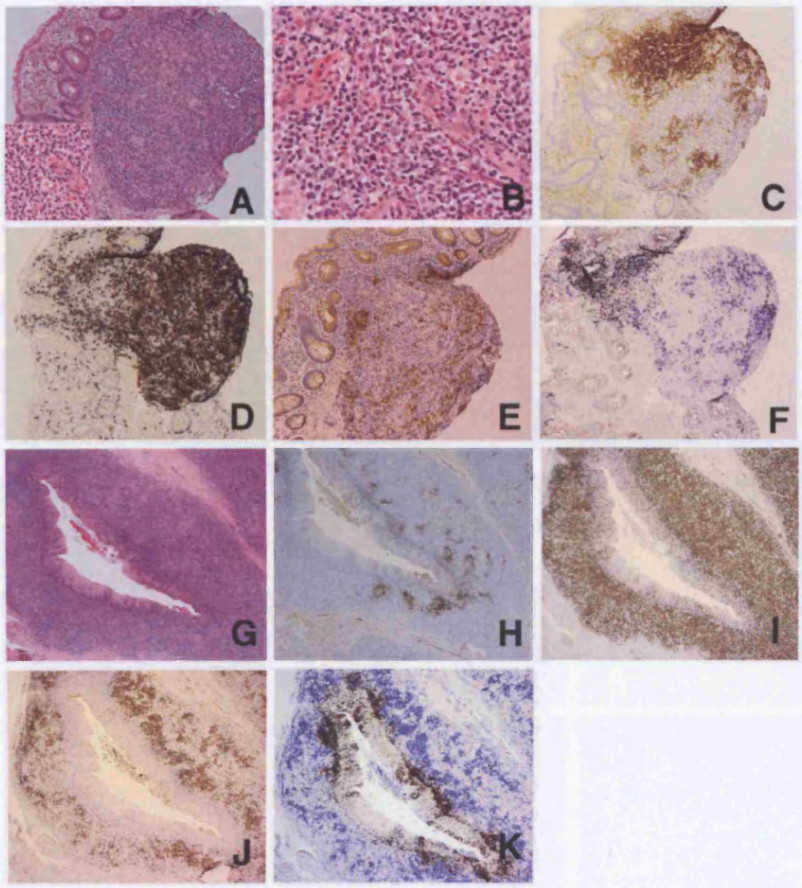

Figure 5.1 Histology, follicular dendritic cell meshwork and CD10 expression in biopsies of caecum and tonsil in case 1.

Panels A-F show the sections of the caecum and panels G-K show sections of the tonsil.
(A and B) Haematoxylin and eosin (H&E) stained section of caecum showing a polymorphous infiltrate with prominent vascularity and clear cells (A, inset and B). (C) CD21 highlights the follicular dendritic cell (FDC) meshwork which is mildly hyperplastic (D) CD3 stain shows numerous T-cells (E) CD10 highlights some lymphoid cells. (F) CD20/CD10 double stain shows many CD10 (blue) positive cells that are negative for CD20 (brown) consistent with T-cells.
(G) H&E stained section of tonsil shows a similar appearance to that seen in the caecal biopsy. (H) CD21 shows subtle FDC expansion. (I) CD3 highlights numerous T-cells. (J) CD10 shows many CD10 positive lymphoid cells. (K) CD20/CD10 double stain shows numerous CD10 positive (blue) cells, similar to the caecal biopsy and consistent with T-cells.

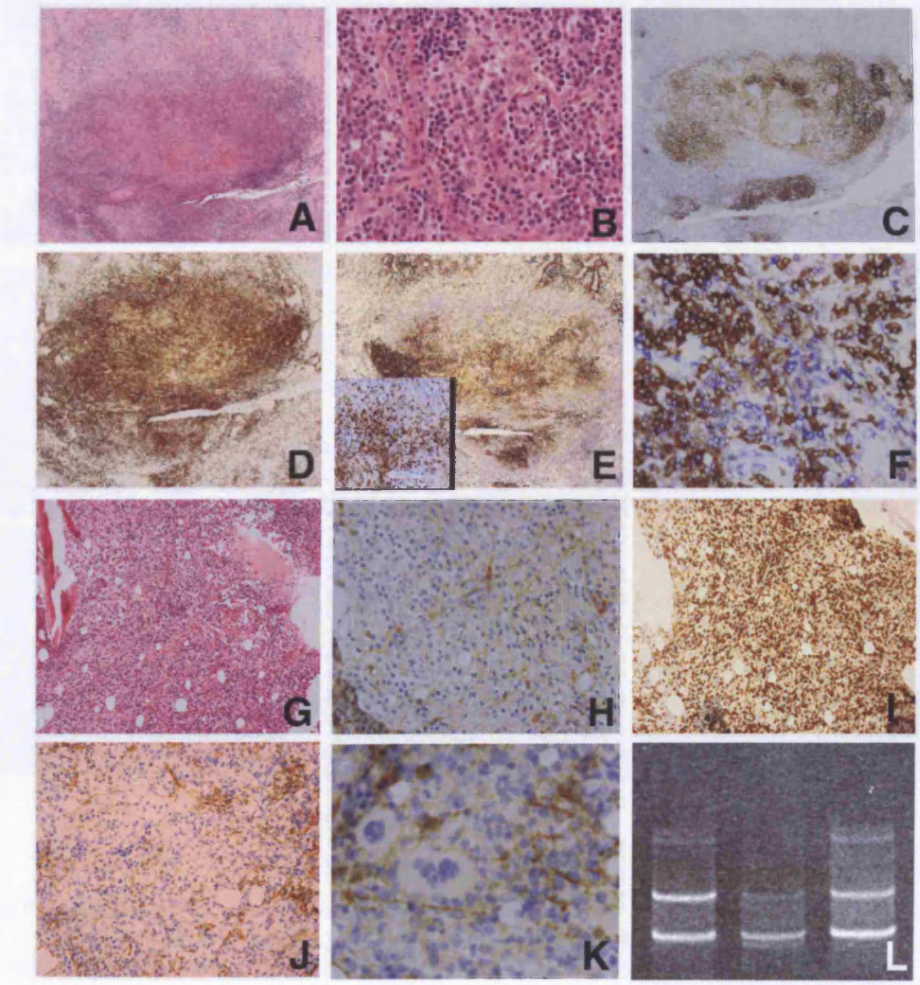

Figure 5.2. Histology, follicular dendritic cell (FDC) meshworks, CD10 positive T-cells and gel image of T-cell receptor gene rearrangement PCR of biopsies of lung and bone marrow of case 2.

Panels A-F shows lung biopsy, panels G-K show sections of bone marrow and panel L shows gel image of T-cell receptor γ gene PCR.

(A and B) Haematoxylin and eosin (H&E) stained section shows a focus with a dense infiltrate which on high power (B) is seen to have a rich vascular network amidst a polymorphous infiltrate that includes eosinophils. (C) CD21 stain of the same focus shows subtle FDC hyperplasia. (D) CD3 shows numerous lymphoid cells in this area. (E, F G) CD10 highlights many lymphoid cells (E), and CD20/CD10 double stain shows numerous CD10 positive (blue) cells, similar to the caecal biopsy and consistent with T-cells. (G)H&E stained section showing involved focus in bone marrow. (H) CD21 stain showing a complete absence of FDC. (I) CD3 higlights numerous T-cells in involved area (J and K) CD10 stain showing the same area highlights neutrophils and stroma, but the lymphoid cells are negative (K).

(L) Gel image of T-cell receptor γ gene PCR of lymph node (left lane), bone marrow (middle) and lung (right) biopsies, each showing 2 dominant bands of identical size.

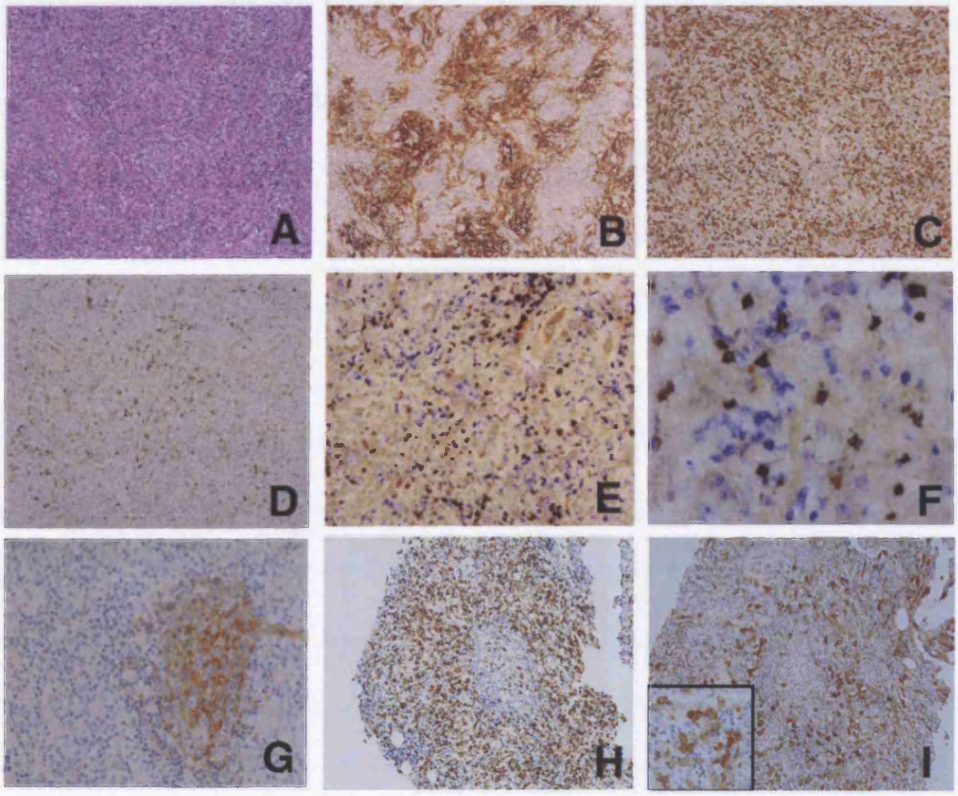

Figure 5.3. Histology, follicular dendritic cell (FDC) meshwork and CD10 positive T-cells in biopsies of lymph node and bone marrow of case 3.

Panels A-F show sections of the lymph node and panels G-I show sections of the bone marrow.
(A) Haematoxylin and eosin stained section shows features of AITL. (B) CD21 highlights the markedly expanded FDC meshwork. (C) CD3 shows numerous T-cells. (D) CD10 highlights many lymphoid cells. (E and F) CD20/CD10 double staining shows many CD10 positive (blue) lymphoid cells that are CD20 (brown) negative, consistent with T-cells.
(G)CD21 highlights a follicle (H) CD3 shows many T-cells. (I) CD10 stain shows many CD10 positive lymphoid cells. I, inset, shows these cells at high power.

5.3 Discussion

5.3.1 Diagnosis of AITL in extranodal sites

The diagnosis of AITL is based on histological examination of a lymph node and requires demonstration of architectural, cytological and immunophenotypic changes often combined with molecular genetic analysis (Frizzera, 2001;Jaffe & Ralfkiaer, 2001a). Of the morphologic features described as being typical for AITL, a polymorphic infiltrate and prominent HEV are rather non-specific, and shared by reactive conditions and other lymphomas. Clear cells, considered characteristic, are present in less than half the cases (see chapter 4). In practice, it is the proliferation of FDCs, best appreciated by immunohistochemistry, and its association with vessels, that is most specific, and ultimately helps to distinguish AITL from PTLu (Jaffe & Ralfkiaer, 2001a;Leung *et al*, 1993). However, this pattern of FDC hyperplasia may be subtle if present at all, and follicular hyperplasia, once thought to exclude AITL, could be a prominent feature (Kojima *et al*, 2001;Ree *et al*, 1998). Thus, except when changes are florid, specific histologic diagnosis can be quite difficult, even on lymph node biopsy, requiring correlation with immunohistochemistry, molecular genetics and importantly the clinical presentation (see chapter 3). This difficulty also questions the diagnostic accuracy of early reports of extranodal involvement, when AITL was believed to be an atypical reactive process, rather than a lymphoma, and diagnosis was purely on morphologic grounds, with no resort to immunohistochemistry or molecular genetic evidence of T-cell clonality. Nevertheless, in both early and more recent reports of visceral involvement by AITL, the histologic features are essentially that of a polymorphous infiltate with prominent vascularity and interstitial periodic acid –Schiff-positive material to follicular hyperplasia with an interfollicular polymorphous infiltrate, similar

to that observed in early lymph node involvement (Ghani & Krause, 1985;Moreb *et al*, 1983;Myers *et al*, 1978;Rosenstein *et al*, 1988;Starke *et al*, 1983;Tobinai *et al*, 1988;Weisenburger *et al*, 1977).

5.3.2 CD10 as a phenotypic marker

In our study, the histology in involved extranodal sites such as lung, nasopharynx, caecum and tonsil showed a similar spectrum of histologic changes, ranging from those that may be mistaken for a reactive lymphoid proliferation to features suggestive of lymphoma, but not specifically AITL. A specific phenotypic or molecular marker would thus be the only means of definite diagnosis of AITL at these sites, in the absence of an accompanying lymph node biopsy. In our study, expression of CD10, a feature shown to be a sensitive and specific marker of neoplastic T-cells in affected lymph nodes, was consistently seen in all involved extranodal sites, except 5/6 involved bone marrow trephines. Its value is especially highlighted in case 2 where the misdiagnosis of reactive (follicular) hyperplasia on lung biopsy may have been averted if CD10 expression had been assessed.

5.3.3 Bone marrow involvement in AITL

Bone marrow is biopsied as a part of staging procedure and is often involved at diagnosis (Dogan & Morice, 2004;Ghani & Krause, 1985). In their series of 8 cases Ghani and Krause (1985) describe focal infiltrates with prominent epithelioid cells imparting a "granulomatoid appearance" as the predominant pattern of involvement. In the same study, paratrabecular involvement described as typical for AITL by Pangalis and co-workers (Pangalis *et al*, 1978) was a rare feature and an increase in vascularity, prominent in involved lymph nodes was seen in only 3 of their cases. In our study

although focal involvement was seen in all involved bone marrow biopsies two thirds (4/6 cases) of which showed a paratrabecular distribution, a "granulomatoid" appearance was seen only in a third of the cases (2/6 cases). An increase in vascularity was also observed only in the latter 2 cases. FDCs, a prominent feature in involved lymph nodes was observed only in case 3 in the form of regressed follicles. In all 6 cases, the morphologic, immunophenotypic and molecular genetic features on bone marrow trephine enabled a diagnosis of PTL, but were not sufficient to subtype further as AITL. CD10 expression, a useful feature in this situation, was however present only in one case (case 3).

5.3.3 Skin involvement

Skin rash is a common symptom at presentation (Siegert *et al*, 1995). It is thought that some of these lesions are immunologically mediated secondary manifestations of systemic disease, perhaps mediated by immunological abnormalities characteristic of AITL rather than direct tumour involvement (Seehafer *et al*, 1980). However, with the advent of molecular methods, it has become apparent that a significant subset of the skin lesions represents direct tumour involvement (Brown *et al*, 2001;Murakami *et al*, 2001). Unfortunately, due to the lack of involved skin biopsies in this series, we were unable to assess the usefulness of CD10 expression at this site.

5.3.4 CD10 expression correlates with the presence of follicular dendritic cell meshwork

CD10, a transmembrane protein with neutral endopeptidase activity is expressed in follicle centre B-cells and is a marker of follicular lymphoma (Dogan *et al*, 2000). In the

latter, CD10, although strongly expressed within the neoplastic follicles, is down regulated in clonally identical interfollicular neoplastic cells (Dogan *et al*, 1998). The expression of CD10 in follicular lymphoma cells may thus be dependent on a signal from the FDC. In vitro studies have shown that highly purified FDCs are able to induce the proliferation of allogeneic T-cells or T-cell lines (Butch *et al*, 1994). There is however no direct evidence for ongoing interactions between the neoplastic cells of T-cell lymphomas and FDCs. Nevertheless, AITL is characterised by an expansion of FDC meshwork (Jaffe & Ralfkiaer, 2001a). In lymph nodes and extranodal sites, CD10 expression correlates well with the presence and distribution of FDCs. This correlation is especially well highlighted in the cases with bone marrow involvement in our study, where CD10 expression in the marrow is confined to the only case associated with FDCs, while in cases 2, 5-8, the neoplastic cells are CD10 negative in the marrow when devoid of associated FDCs, but CD10 positive in other involved sites that harboured FDCs. This suggests an analogy with follicular lymphoma where despite clonal identity, there is down regulation of CD10 in neoplastic cells that lack association with FDCs and indicate a possible role of FDCs in this phenomenon. In support of this theory, in a recent study, Kim *et al*, have shown that FDCs and endothelial cells in AITL express FasL whereas the CD10 expressing tumour T-cells express Fas (CD95) and caspase 3, indicating Fas-FasL interaction between the neoplastic T-cell and the FDC and suggest that the follicular milieu is necessary for CD10 expression by tumour cells, a feature that may play a functional role in regulating apoptosis (Kim *et al*, 2002a).

5.3.5 Concluding remarks

In AITL, expression of CD10 by neoplastic T-cells is maintained in most involved extranodal sites and shows good correlation with the presence/distribution of FDCs. This immunophenotypic feature may thus be used to make a diagnosis of AITL in an extranodal site, even in the absence of accompanying lymph node histology.

Chapter 6

HISTOLOGIC PROGRESSION OF ANGIOIMMUNOBLASTIC T-CELL LYMPHOMA

6.1 Introduction

As was discussed in chapter 1, AITL was initially believed to be an atypical reactive process, "AILD" (Frizzera *et al*, 1974;Lennert, 1979;Lukes & Tindle, 1975) a concept that was refuted by molecular genetic evidence of a monoclonal T-cell proliferation in most cases (Feller *et al*, 1988;Lipford *et al*, 1987;Weiss *et al*, 1986). Although it is now universally accepted as a subtype of PTL (Harris *et al*, 2000), there is still a debate as to whether the T-cell lymphoma arises de novo, or in the background of an oligo-clonal pre-neoplastic state. The clonal expansion of by-stander EBV infected B-cells and the occurrence of an EBV-associated B-cell lympho-proliferation in some cases causes confusion with regard to lineage assignment, diagnosis, and prognostication (Smith *et al*, 2000). The prognosis in AITL is dismal, with a median survival of less than 3-years following diagnosis (Pautier *et al*, 1999;Pellatt *et al*, 2002;Siegert *et al*, 1992). Despite this aggressive course, the cause of death in many cases is due to the consequences of secondary immune-deficiency, rather than tumour bulk (Siegert *et al*, 1995). As the disease is uncommon, and the biology poorly understood, there are no uniform treatment protocols and treatment remains empirical. There are a few clinical trials that are being set up, each targeting different aspects of the disease - immune-modulation with cyclosporine A in relapsed AITL (Eastern Coorporative Oncology Group, 2003) and Rituximab with CHOP in cases of AITL with a prominent B-cell component (Joly *et al*,

2004). Histologic progression of the disease is poorly studied and documentation is limited to a few isolated case reports and small case series (Abruzzo *et al*, 1993;Bauer *et al*, 1982;Joly *et al*, 2004;Knecht *et al*, 1995;Matsue *et al*, 1998;Nathwani *et al*, 1978;Park *et al*, 2002;Pirker *et al*, 1986;Zettl *et al*, 2002).

The aim of this part of the study was to evaluate the histology in sequential biopsies in a large series of patients in order to correlate histology with the evolution of the disease.

6.2 Results

6.2.1 Tissues used in this study

Twenty-nine cases of AITL where sequential biopsies were available were reviewed and the diagnosis confirmed on clinical, histological, immunophenotypic and genetic criteria, the data are summarized in Table 6.1.

6.2.2 Clinical features

There were 17 males and 12 females who were between the ages of 26 and 80 years at presentation. In most cases (where staging information was available) they presented with disseminated disease (Table 6.1). In 28 of the 29 cases, a lymph node was biopsied at presentation while in one case a lung biopsy was obtained for diagnosis. In all 29 cases however, the initial diagnosis of AITL was made on lymph node biopsy (Table 6.1).

Table 6.1. Summary of clinical details, histology, molecular genetics, therapy and outcome.

Case No	Age/Sex	Month	Biopsy site	Histology	TCR	Ig H	Stage	Treatment	Outcome
1	42/M	0	LN	AITL-1	MC	PC	IVB	None	N/A
		2		AITL-3	MC*	PC	IVB	N/Av	N/Av
									N/Av
2	69/M	0	LN	AITL-1	MC	PC	IIA	None	N/A
		23	LN	AITL-3	MC*	PC	IIIB	Et+P	NR
		26	SK	AITL	MC*	PC	IVB		
									DOD, 26 months
3	74/F	0	LU	AITL-1	MC	PC	ND	None	
		18	LN	AITL-3	MC*	PC	IVB	N/Av	
									N/Av
4	28/M	0	LN	AITL-3	N/Amp	N/Amp	IIA	CHOP	CR
		20	LN	AITL-3	MC	PC	IIIA	ESHAP	
								A-BMT	PR
		22	BM	AI TL	N/Amp	N/Amp	IVB	ESHAP	NR
								G	PR
		24	BM	EBV-DLBCL	N/Amp	N/Amp	IVB	G	NR
									Died, 27 months
5	60/F	0	LN	AITL-3	MC	PC	IVA	CHOP	CR
		8	LN	AITL-3 & EBV-DLBCL	MC*	MC	IVB	CHOP, Et	NR
									Died, 11 months
6	60/M	0	LN	AITL-3	MC	PC	IIIB	CHOP	CR
		13	LN	AITL-3	MC*	MC	IA	Et, SCT	CR
		37	SA	AITL	MC*	PC	IIA	N/Av	NR
		37	BM	AITL	ND	ND	IV	N/Av	NR
									Died 38 months
7	79/F	0	LN	AITL-3	MC	PC	N/Av	N/Av	
		63	LN	EBV-CHL	PC	PC	N/Av	N/Av	
									N/Av
8	67/F	0	LN	AITL-3	MC	ND	IVB	CHOP	CR
		60	LN	EBV-CHL	PC	PC	N/Av	ABVD	CR
									A&W 72 months
9	62/M	0	LN	AITL-3	MC	PC	IIIB	CEOP	CR
		28	LN	AITL-3	MC*	PC	IIIB	Et+N	CR
		36	SK	AITL	MC*	ND	IVB	M +If + Et	NR
									Died, 36 months

Table 6.1 continued from previous page

Case No	Age/Sex	Month	Biopsy site	Histology	TCR	Ig H	Stage	Treatment	Outcome
10	33/F	0	LN	AITL[##]	N/Av	N/Av	N/Av	N/Av	
		11	CAE	AITL[$]	OC	PC	IVB	N/Av	
		11	TON	AITL-3	N/Av	N/Av	IVB	N/Av	
									N/Av
11	78/F	0	LN	AITL-3 & EBV-DLBCL	MC	MC	IIIA	Thal	PR
		3	LN	AITL2	MC*	PC	IIIA	None	PR
									AWD, 12 months
12	58/F	0	LN	AITL-3	OC	PC	IIIB	CHOP	CR
		58	LN	AITL-3	MC**	PC	IVB	ESHAP	NR
									Died, 60 months
13	80/F	0	LN	AITL-2	MC	N/Amp	N/Av	Palliative	N/A
		24	GA	PTL	Indet	PC	N/Av	Palliative	N/A
									N/Av
14	26/M	0	LN	AITL-2	N/Amp	PC	IVB	CHOP	PR
		6	LN	AITL-2	N/Amp	PC	IVB	ABVD, A- BMT	CR
									Died 26 months
15	33/M	0	LN	AITL-3	OC	PC	IVB	CHOP	CR
		12	LN	AITL-3	OC*	PC	IVB	CHOP	PR
									Died 12 months
16	73/M	0	LN	AITL-3	MC	PC	N/Av	N/Av	N/Av
		22	LN	AITL-3	MC	PC	N/Av	N/Av	N/Av
									N/Av
17	49/F	0	LN	AITL-3	PC	PC	N/Av	N/Av	N/Av
		17	LN	AITL-3	Indet	Indet	N/Av	N/Av	
		17	SK	AITL[$]	Indet	Indet	N/Av	N/Av	
									Died 21 months
18	69/M	0	LN	AITL-3	N/Av	N/Av	IIIB	P	PR
		8	LN	EBV-DLBCL	MC	MC	IA	Multi-CT	CR
		15	LN	AITL-3	mc*	PC	IIIA	Multi-CT	PR
									AWD 68 Months
19	32/M	0	LN	AITL-1/2	N/Av	N/Av	IIIA	None	N/A
		7	LN	AITL-2	MC	PC	IIIB	CVP	PR
		23	LN	AITL-3	MC	PC	IIIB	ACVBP,BMT	CR
		98	LN	AITL-1	N/Av	N/Av	IIIB	P	PR
									AWD, 119 months

Table 6.1 continued from previous page

Case No	Age/Sex	Month	Biopsy site	Histology	TCR	Ig H	Stage	Treatment	Outcome
20	76/M	0	LN	AITL-3	MC	mc	IIIB	P	PR
		3	LN	AITL-3	MC*	mc*	IIIB	N/Av	N/Av
		3	SK	AITL$	MC*	mc*	IIIB	N/Av	N/Av
									N/Av
21	64/M	0	LN	AITL-1	PC	PC	IVA	None	N/A
		5	LN	AITL-2	MC	PC	IVA	None	N/A
		37	LN	N-DX	N/Av	N/Av	IVB	N/Av	N/Av
22	55/F	0	LN	AITL-3	MC	PC	IVA	Multi-CT, A-BMT	PR NR
		10	SK	AITL$	MC	N/Av	IVA	P	Died, 13 months
23	49/M	0	LN	AITL-3	N/Av	N/Av	IVB	Multi-CT,	
		0	SK	AITL$	N/Av	N/Av		A-BMT	PR
		12	OR	AITL$	N/Av	N/Av	IVB	P	NR
									Died, 17 months
24	67/F	0	LN	AITL-3	MC	N/Av	IIIA	Multi-CT	CR
		42	LN	AITL-2	MC	MC	IA	CT	N/Av
									N/Av
25	43/F	0	LN	AITL-3	N/Av	N/Av	N/Av	P	CR
		228	SK	AITL$	MC	PC	IVA	C + P	PR
		228	WAL	AITL$	MC	PC			
		228	BLD	AITL$	MC*	PC			
								CHOP-R	Relapse 1y following Rx, AWD, 247 months
26	72/M	0	LN	AITL-3	MC	MC#	IIIB	N/Av	CR
		84	LN	EBV-DLBCL	PC	MC**	IA	N/Av	N/Av
									N/Av
27	N/Av/M	0	LN	AITL-1	N/Av	N/Av	IIIA	P	CR
		168	LN	AITL-3	MC	PC	IIIB	N/Av	N/Av
									N/Av
28	52/M	0	LN	AITL-3	N/Av	N/Av	IIIB	Multi-CT	CR
		8	LN	EBVneg DLBCL	N/Av	N/Av	N/Av	Multi-CT-R	CR
									A&W 24 months
29	54/F	0	LN	AITL-3	MC	PC	IB	P	CR
		22	LN	AITL-2	MC*	PC	IB	None	N/A AWD 25 months

[$] Not assigned a "pattern" because site of involvement is extranodal
[*] Same size band as previous biopsies
[**] Different size band/bands to previous biopsies
[#] Weak band
[##] Biopsy diagnosed elsewhere; not available for review

Abbreviations: F, female; M, male; LN, lymph node; SK, skin, LU, lung; BM, bone marrow; SA, salivary gland; CAE, caecum; TON, tonsil; GA, gastric; OR, oral; WAL, waldeyer's ring; BLD, blood; AITL, angioimmunoblastic T-cell lymphoma; AITL-1, angioimmunoblastic T-cell lymphoma, pattern 1; AITL-2, angioimmunoblastic T-cell lymphoma, pattern 2; AITL, pattern 3, angioimmunoblastic T-cell lymphoma, pattern 3; EBV, Epstein Barr virus; DLBCL, diffuse large B-cell lymphoma; neg, negative; CHL, classical Hodgkin lymphoma; MC, monoclonal; PC, polyclonal; mc, oligoclonal with a weak dominant band; OC, oligoclonal;NDx, not diagnostic; ND, not done; N/Amp, not amplifiable; indet, indeterminate; N/A, not applicable; N/Av, not available; E (in CEOP regimen), epirubicin; Et, etoposide; G, gemcitabine; P, prednisolone; C, cyclophosphamide; H, doxorubicin; O, vincristine, R, Rituximab; A, adriamycin; B, bleomycin; V, vinblastine; D, dacarbazine; ESHAP, Etoposide, methyl prednisolone, cytarabin (ara-C) and cisplatin; N, novantrone; M, methotrexate; If, ifosphamide; Thal, thalidomide; B, bleomycin; CT, chemotherapy, details not available; multi-CT, multi-agent chemotherapy, details not available; SCT-stem cell transplant; BMT, bone marrow transplant; A-BMT, autologous bone marrow transplant; CR, complete remission; PR, partial response; NR, no response; A&W, alive and well; AWD, alive with disease; y, year; Rx, treatment

6.2.3 Histology, immunohistochemistry and EBER-ISH

The histological diagnosis for each biopsy is given in Table 6.1 and the summary of EBER-ISH results are given in Table 6.2.

6.2.3.1 "Pattern I" on initial biopsy to more typical AITL (patterns II/III) on follow up

In 6 cases (cases 1-3, 13, 21 and 27) the initial histology showed a predominantly "pattern I" morphology (Figure 6.1), with hyperplastic follicles (see chapter 3) while the histology on follow up biopsy showed a "pattern II" or "pattern III" appearance with effaced architecture and either regressed follicles (pattern II) or no identifiable follicles (pattern III) (Figure 6.2). The FDC meshworks, highlighted by CD21 immunohistochemistry was as described for patterns I, II and III in chapter 3. The time interval between the 2 biopsies ranged from 2 to 23 months in 5 of these 6 cases, but was 168 months in one of the cases (case 27). In one case (case 19), the histology showed a "pattern I" appearance on initial biopsy and "typical" AITL with patterns II and III respectively at 1st and 2nd relapse, but the biopsy at 3rd relapse, 98 months after initial biopsy showed "pattern I" morphology with hyperplastic follicles.

6.2.3.2 "Typical" AITL on initial biopsy and follow up lymph node biopsies

In 10 cases (cases 4, 5, 6, 9, 12, 14, 15, 17 and 20), the initial and follow up biopsies showed the same appearance of "typical" AITL. Nine of these 10 cases showed pattern III histology and 1 case (case 14) showed a pattern II appearance on initial and follow up

biopsies. In 3 other cases (cases 11, 24 and 29) the histology showed a pattern III appearance on initial biopsy and pattern II in the second biopsy. In case 11, the vascularity was less pronounced in the second biopsy (a feature demonstrated better by CD34 immunohistochemistry – see Figure 6.4I and J)

6.2.3.3 Relapse of AITL (and biopsy) involving extranodal site

In 12 cases, the follow up biopsies of involved extranodal sites showed features of involvement by a T-cell lymphoma. In some sites (e.g. caecum and tonsil in case 10) the histology showed prominent vascularity and clear cells suggestive of AITL, but in others (e.g. skin biopsies in cases 2, 9, 17, 20, 22, 23 and 25), the features were non-specific. In one case (case 13) the patient relapsed 24 months following initial biopsy with gastric involvement, and the gastric biopsy showed a diffuse monomorphic infiltrate of highly atypical CD3+ T-cells, not present in the initial biopsy, and the diagnosis of PTL (rather than AITL) was made (Figure 6.3).

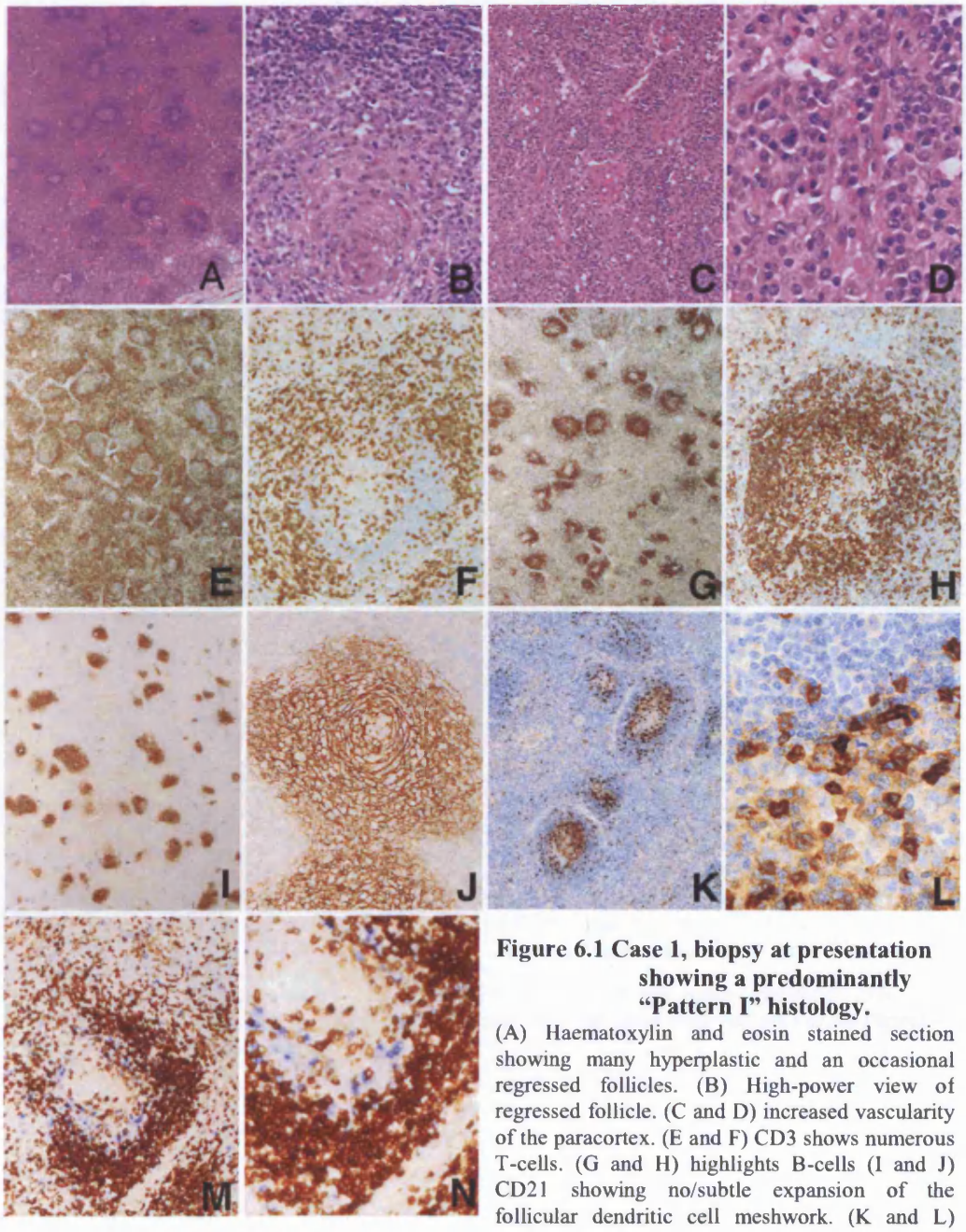

Figure 6.1 Case 1, biopsy at presentation showing a predominantly "Pattern I" histology.

(A) Haematoxylin and eosin stained section showing many hyperplastic and an occasional regressed follicles. (B) High-power view of regressed follicle. (C and D) increased vascularity of the paracortex. (E and F) CD3 shows numerous T-cells. (G and H) highlights B-cells (I and J) CD21 showing no/subtle expansion of the follicular dendritic cell meshwork. (K and L) CD10 staining shows lighter staining germinal center B-cells and darker, crisply staining neoplastic T-cells. (M and N) CD79a/CD10 double staining shows CD10 positive (blue) lymphoid cells that are CD79a (brown) negative, consistent with T-cells.

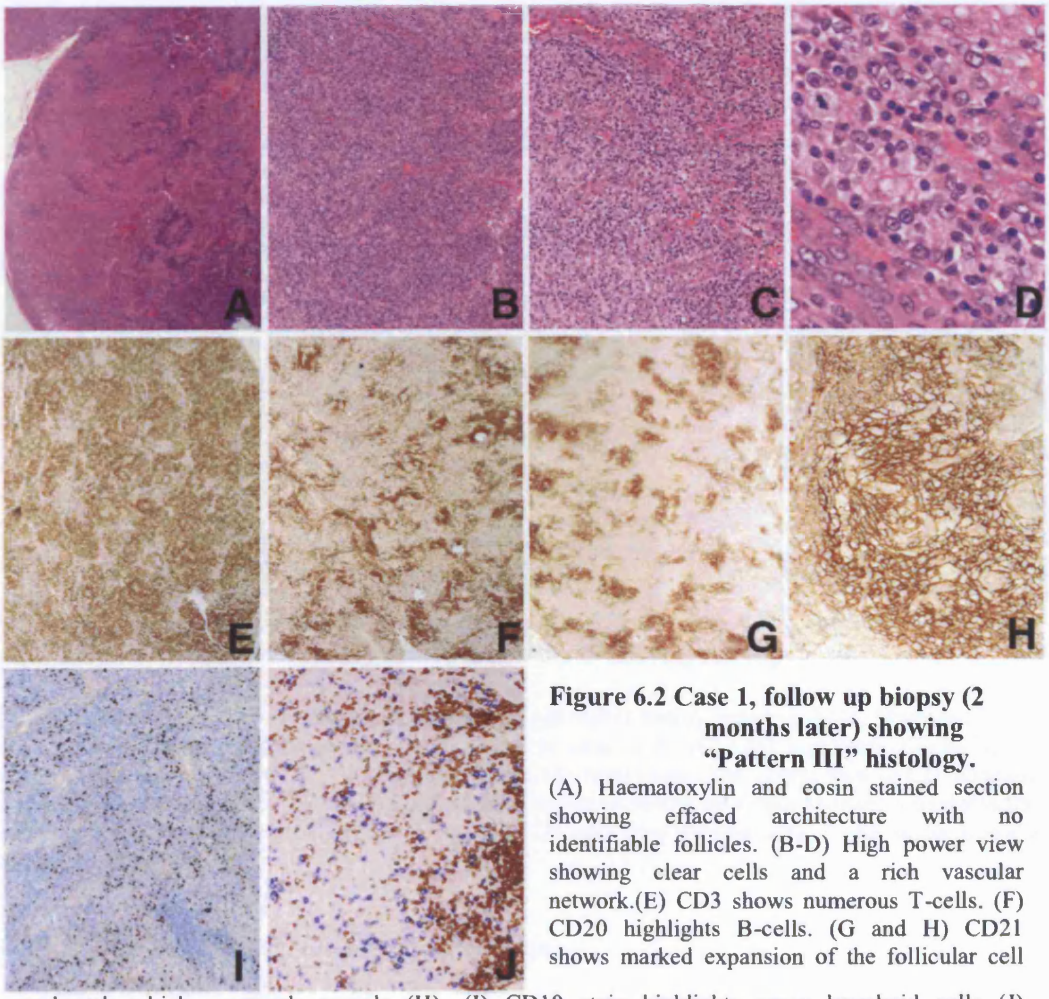

Figure 6.2 Case 1, follow up biopsy (2 months later) showing "Pattern III" histology.

(A) Haematoxylin and eosin stained section showing effaced architecture with no identifiable follicles. (B-D) High power view showing clear cells and a rich vascular network.(E) CD3 shows numerous T-cells. (F) CD20 highlights B-cells. (G and H) CD21 shows marked expansion of the follicular cell meshwork which surrounds vessels (H). (I) CD10 stain highlights many lymphoid cells (J) CD79a/CD10 double stain shows many CD10 positive (blue) lymphoid cells that are CD79a (brown) negative, consistent with T-cells

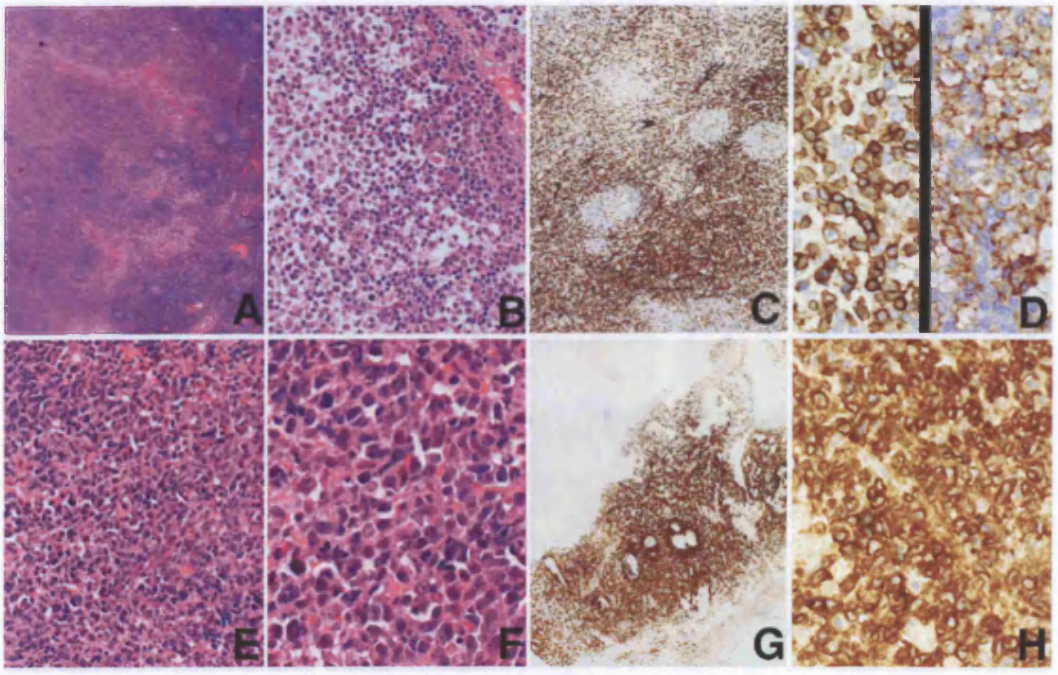

Figure 6.3. Initial and follow up biopsies from case 13, showing AITL (panels A-D) and Peripheral T-cell lymphoma (panels E-H), respectively.

(A) Haematoxylin and eosin (H&E) stained section of initial biopsy showing AITL "Pattern III". (B) High power view of initial biopsy showing clusters of clear cells. (C) CD3 highlights T-cells (D, left panel) high power view of CD3 stain showing T-cells, (D, right panel) high power view of CD10 staining highlights neoplastic T-cells. (E and F) H&E stained section of follow up biopsy showing a monomorphic infiltrate of large atypical lymphoid cells (G and H) CD3 highlights the neoplastic T-cells in the follow up biopsy.

6.2.3.4 EBV-associated B-cell proliferations

The results of EBER-ISH are summarized in Table 6.2. Seven of the 29 cases (24%) in this series showed evidence of EBV-associated B-cell "lymphomas" (5, DLBCL; 2 CHL) and 3 cases had a dominant B-cell clone in the presence of prominent EBV-infected B-cells.

6.2.3.4.1 *EBV-associated DLBCL*

One case (case 11) showed histological features of AITL and co-existent numerous EBV-associated large B-cells amounting to DLBCL at initial presentation (Figure 6.4, left panels, A, C, E, G, I, K). The DLBCL in this case had regressed with a complete

absence of EBER-ISH (in situ hybridization) positive cells in the follow up biopsy (Figure 6.4L). Four cases (cases 4, 5, 18 and 26) that showed only AITL on initial biopsy, showed DLBCL on follow up biopsy (Figure 6.5). In 2 of these cases (cases 4 and 5) where EBER-ISH was performed on the biopsy pre-dating the development of DLBCL [3rd biopsy (bone marrow) in case 4 and initial biopsy in case 5] the latter showed a prominence of EBER-ISH positive cells. Cases 5 and 18 had co-existent AITL and EBV-associated DLBCL in the same biopsy (Figure 6.5F-H). The time interval between biopsies (the preceding biopsy and the biopsy showing EBV-associated DLBCL) ranged from 2-8 months in 3 cases but was 84 months in one case (case 26). In one of the cases (case 18), following multi-agent chemotherapy and complete remission, the patient relapsed again 7 months later with AITL and no evidence of DLBCL.

6.2.3.4.2 EBV- associated Classical Hodgkin lymphoma (CHL)

Two cases (cases 7 and 8) developed EBV-associated CHL 5-years after complete remission was achieved following initial diagnosis and treatment (Figure 6.6). The Reed-Sternberg cells in these cases were LMP-1 (immunohistochemistry) and EBER (in situ hybridization) positive (Figure 5.6K). Although the presence of a polymophous reactive background in

CHL made interpretation a little difficult, there were no histological, immunophenotypic or molecular genetic evidence of AITL in these follow up biopsies.

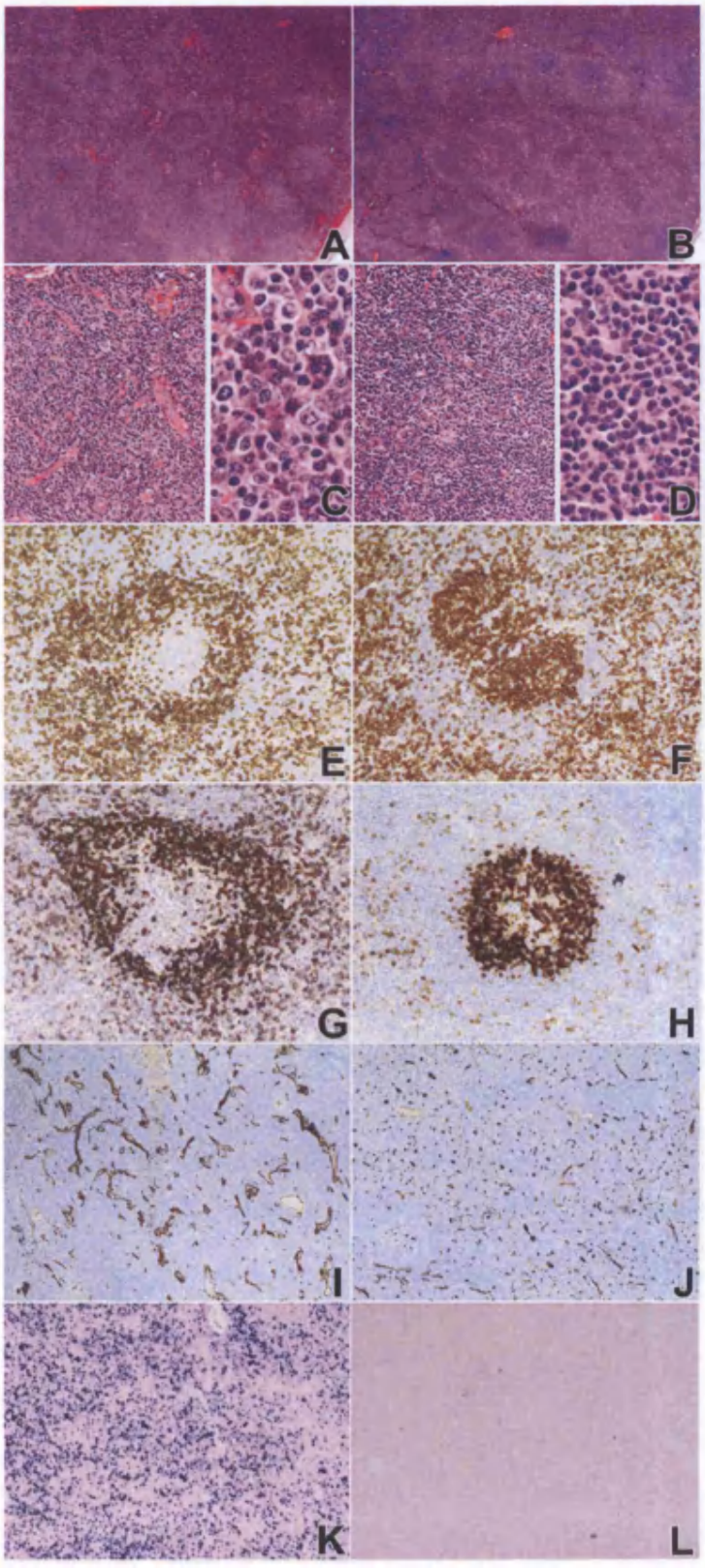

Figure 6.4 Biopsies from case 11 showing AITL "Pattern III" histology and co-existent EBV positive diffuse large B-cell lymphoma in the initial biopsy and "Pattern II" with regression of the EBV-positive lympho-proliferation in the follow up biopsy, 10 weeks of Thalidomide treatment.

Left panels (A,C,E,G,I and K) show the biopsy before treatment and the right panels (B,D,F,H,J and L) show the biopsy after treatment. (A and B) Haematoxylin and eosin stained section, low power view of excised lymph nodes; C and D) high power view highlighting subtle changes in vascular architecture and cellular morphology; E and F) CD3 immunostaining and G and H) CD10 immunostaining showing the differences in distribution of neoplastic T-cells after treatment; I and J) CD34 immunostaining highlighting the changes in vascular architecture after treatment; K and L) EBER in-situ hybridisation showing disappearance of EBV infected B-cell proliferation after treatment. The positive cells show dark blue/purple nuclear staining.

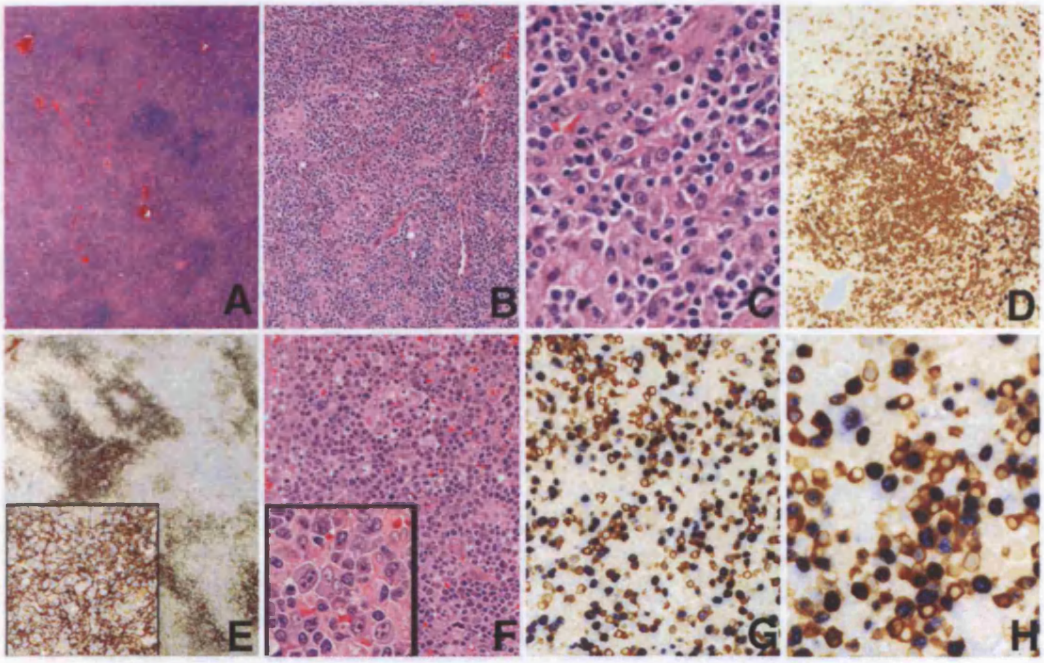

Figure 6.5 Case 5, the initial (Panels A-E) biopsy showing AITL, "pattern III and follow up (F-H) biopsies showing AITL pattern III in the initial biopsy and AITL pattern III with EBV-associated DLBCL in the follow up biopsy.

(A-C) Haematoxylin and eosin (H&E) section showing "pattern III" morphology with prominent vascularity and clear cells (high power, panel C). (D) double EBER-in situ hybridization (EBER-ISH) and CD79a immunohistochemistry highlighting EBER-positive B-cells. (E) CD21 highlights the expanded follicular dendritic cell meshwork which surrounds vessels (inset)/ (F) H&E stained section of follow up biopsy with many large atypical cells, some of which have a Reed-Sternberg-like morphology (inset). (G and H). Double EBER-ISH, CD79a immunohistochemistry showing numerous EBER-positive B-cells consistent with diffuse large B-cell lymphoma

6.2.3.5 EBV-negative DLBCL on follow up

One case (case 28) showed features of DLBCL (EBV-negative) on follow up biopsy 8 months after the initial diagnosis and after complete remission was achieved with multi-agent chemotherapy.

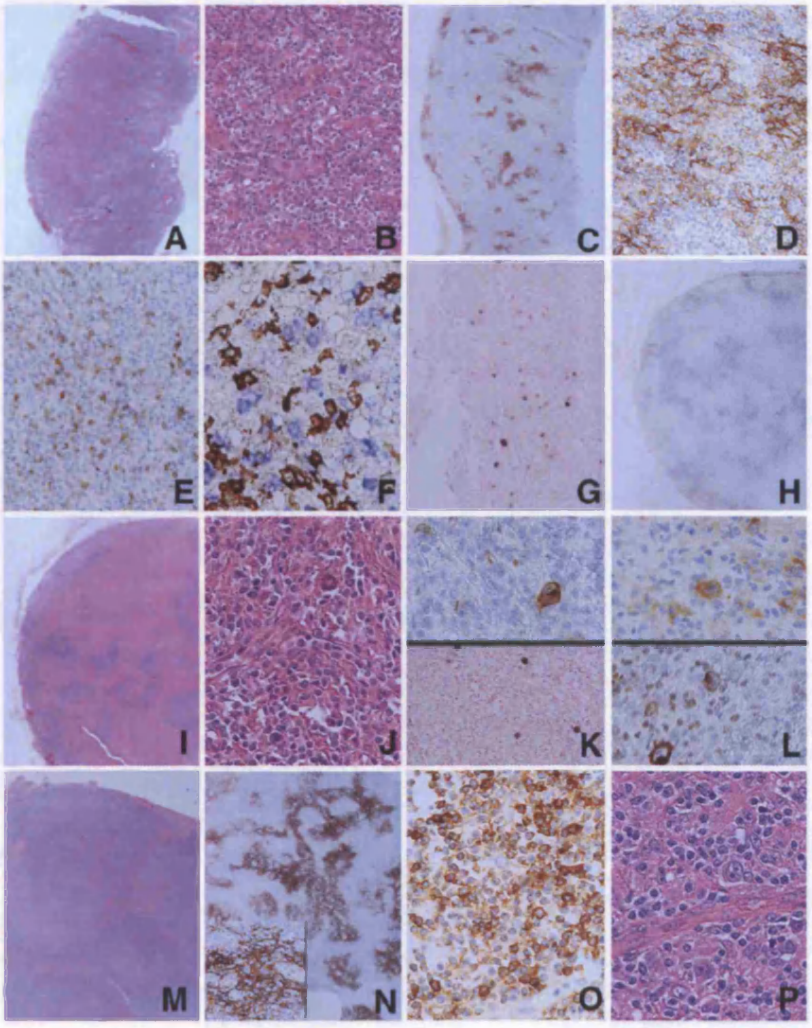

Figure 6.6 Cases 7 and 8 showing progression from AITL (initial biopsy) to classical Hodgkin lymphoma (follow up biopsy 5-years later).

Panels A-L show the biopsies from case 7; panels A-G, show the initial biopsy and panels H-L show the follow up biopsy. Panels M-P show the biopsies from case 8; panels M-O show the initial biopsy and panel P shows the follow up biopsy.
(A and B) Haematoxylin and eosin (H&E) stained sections of the initial biopsy of case 7 showing AITL, "Pattern III". Panel B shows a high power view with clusters of clear cells amidst a rich vascular network. (C and D) CD21 staining highlights the marked expansion of the follicular dendritic cell (FDC) meshwork, and shows that it surrounds vessels (D). (E) CD10 highlights neoplastic T-cells. (F) CD20/CD10 double staining shows CD10 positive lymphoid cells, negative for CD20 and consistent with T-cells. (G) EBER in situ hybridisation (EBER-ISH) highlights scattered EBV-infected cells. (H) CD21 staining of the follow up biopsy of case 8 shows absence of FDC. (I and J) H&E stained sections of the same biopsy shows features consistent with classical Hodgkin lymphoma (CHL), with mummified and Reed-Sternberg (RS) cells (J). (K, upper panel) LMP-1 and (K, lower panel) EBER-ISH highlight EBV-infected RS cells. (L, upper panel) CD30 and (L, lower panel) CD15 positive RS cells. (M) H&E stained section of the initial biopsy of case 8 showing "Pattern III" AITL. (N) CD21 highlights the expanded FDC meshwork that encircles vessels (inset). (O) CD10 highlights the neoplastic T-cells. (P) H&E stained section showing CHL with typical RS cells in a polymorphous background.

6.2.3.6 CD10 expression by neoplastic T-cells

The results of CD10 immunostaining are given in Table 6.2. The neoplastic T-cells expressed CD10 in 21 of 24 cases that were immunostained for CD10. If involved by AITL, CD10 expression was maintained in the initial biopsy and follow up lymph node biopsies in all but 2 cases (cases 3 and 27) where material was available for assessment. In cases 3 and 27, although the initial biopsies expressed CD10, the follow up lymph node biopsies were CD10 negative.

6.2.3.6.1 *"Pattern I" on initial biopsy to more typical AITL (patterns II/III) on follow up*

The initial as well as follow up biopsies in 5 cases (cases 1, 2, 3, 19 and 27) showing pattern I histology on the initial biopsy and more typical AITL on follow up were investigated for CD10 expression. In 3 cases (cases 1, 2 and 19), CD10 expressing neoplastic T-cells were concentrated in the vicinity of the hyperplastic follicles on initial biopsy, and increased in number, were more diffusely distributed, but concentrated around the expanded FDC meshwork in the later biopsies showing patterns II and III morphology. In case 19 where the biopsy at 3rd relapse showed a pattern I appearance, the CD10 positive neoplastic cells were present predominantly in the vicinity of the follicles. In 2 cases (cases 3 and 27), although the initial biopsy was CD10 positive, the follow up biopsy was CD10 negative. In case 3, the initial (lung) biopsy, which was misdiagnosed as reactive showed CD10 expression by the neoplastic T-cells, but the subsequent poorly fixed diagnostic lymph node biopsy showing pattern III histology was CD10 negative. In case 27, although the neoplastic T-cells in the initial lymph node

biopsy were CD10 positive, the lymph node biopsy at relapse 168 months later was CD10 negative.

In one of the cases (case 21) although the neoplastic T-cells expressed CD10 in the initial biopsy, due to unavailability of spare material, immunohistochemistry for CD10 was not performed in the follow up biopsy.

6.2.3.6.2. *"Typical" AITL on initial biopsy and follow up lymph node biopsies*

Ten cases (cases 4-6, 9, 11, 12, 14, 15, 20, and 24) that showed "typical" AITL on initial and follow up biopsies were investigated for CD10 expression.

CD10 expression by the neoplastic T-cells was maintained in both initial and follow up biopsies in 7 of the 10 cases (cases 4, 6, 9, 11, 12, 14, and 15) investigated. In 6 of the 7 cases the distribution and proportion of CD10 positive T-cells were similar in initial and follow up biopsies, while in one case (case 9) the follow up biopsy which was poorly fixed showed a marked reduction in the number of CD10 expressing tumour cells. In one case (case 11), although there was no sizeable reduction in the number, the tumour cells which were more diffusely distributed in the initial biopsy (pattern III histology), were more confined to the vicinity of the follicles in the post-treatment biopsy (pattern II histology) (Figure 6.4H).

In case 20, although CD10 expression was present in the follow up biopsy, this could not be assessed in the initial biopsy due to lack of access to spare material.

In 2 of the cases (case 5 and 24), the neoplastic T-cells failed to express CD10 in both initial and follow up biopsies.

6.2.3.6.3. *Relapse of AITL (and biopsy) involving extranodal site*

In case 9, CD10 expression was observed in both tonsil and caecum at relapse. Due to technical reasons, it was not possible to demonstrate CD10 expression in skin biopsies (cases 3 and 9) showing involvement by AITL. In case 25, CD10 expression was observed in the initial lymph node biopsy, but was not investigated for in the Waldeyer's ring biopsy involved at relapse 228 months later. Nevertheless at this time the presence of CD10 positive T-cells in the peripheral blood was detected on flow cytometry in this case. In one of the cases (case 13), the neoplastic T-cells in the initial biopsy (showing AITL) were CD10 positive, but in the follow up gastric biopsy showing a monomorphic proliferation of large atypical T-cells (Figure 6.3E and F), the latter were CD10 negative.

6.2.4 PCR for T-cell receptor γ chain (TCR-γ) gene rearrangement and immunoglobulin heavy chain (IgH) gene rearrangement

6.2.4.1 PCR for TCR-γ gene rearrangement

The results of PCR for TCR-γ gene rearrangement are summarized in Table 6.1. In 25 cases, T-cell receptor γ chain gene PCR gave a monoclonal/oligoclonal pattern in at least one of the biopsies (Table 1). One case (case 17) gave a polyclonal pattern on initial biopsy and an indeterminate result on follow up biopsies, while one case (case 14) failed to amplify due to poor quality of DNA. In two further cases (cases 23 and 28), the molecular genetics results were not available. In 12 of the 18 cases, where PCR was successful and AITL was present in both initial and follow up biopsies, the band/bands

in the initial and follow up biopsies were identical in size. In 4 cases the PCR products of both initial and follow up biopsies could not be compared. In one case (case 12) the initial biopsy had showed an oligoclonal pattern, while the biopsy at relapse showed a monoclonal pattern, with a band size that did not correspond to any of the bands of the previous biopsy. In case 21 the initial biopsy showed a polyclonal pattern while the follow up biopsy showed a monoclonal pattern.

6.2.4.2 PCR for IgH gene rearrangement

The results of PCR for IgH gene rearrangement are summarized in Table 7.1

6.2.4.2.1 *EBV-associated DLBCL*

In case 11, the initial biopsy showed features of AITL and a numerous EBV-infected (EBER-positive) B-cell cells with molecular genetic evidence of a dominant B-cell clone i.e. AITL with EBV-associated DLBCL. The post-treatment biopsy, with features of AITL and a complete absence of EBER-ISH positive cells, showed a polyclonal Ig heavy chain gene PCR result.

In 3 of the 4 cases (cases 5, 18 and 26) that were complicated by EBV-associated DLBCL on follow up biopsies, a monoclonal B-cell population was detected by PCR in the biopsy showing DLBCL. In one of these cases (case 26) the preceding biopsy that had no features of DLBCL gave rise to a weak monoclonal band, differing in size to that of the subsequent biopsy. In one case (case 5), although the biopsy showing DLBCL had a dominant B-cell clone, the preceding biopsy, which showed many scattered EBV-infected cells (EBER-ISH positive), showed a polyclonal pattern by PCR.

Table 6.2 CD10 expression and EBER in situ hybridization

	Biopsy	Diagnosis	CD10 positve T-cells	EBER-ISH
Case 1	1st biopsy (lymph node)	AITL-1	Positive	+++
	2nd biopsy (lymph node)	AITL-3	Positive	++
Case 2	1st biopsy (lymph node)	AITL-1	Positive	++
	2nd biopsy (lymph node)	AITL-3	Positive	Negative
	3rd biopsy (skin)	AITL*	Not interpretable	Not done
Case 3	1st biopsy (lung)	AITL-1	Positive	Not done
	2nd biopsy (lymph node)	AITL-3	Negative	Not done
Case 4	1st biopsy (lymph node)	AITL-3	Positive	Not done
	2nd biopsy (lymph node)	AITL-3	Positive	Not done
	3rd biopsy (bone marrow)	AITL*	Not done	+
	4th biopsy (bone marrow)	EBV-DLBCL	Negative	Diffuse sheets of positive cells
Case 5	1st biopsy (lymph node)	AITL-3	Negative	++
	2nd biopsy (lymph node)	AITL & EBV-DLBCL	Negative	++++
Case 6	1st biopsy (lymph node)	AITL-3	Positive	++
	2nd biopsy (lymph node)	AITL-3	Positive	++
	3rd biopsy (parotid)	AITL*	Negative	+
	4th biopsy (bone marrow)	AITL*	Not done	Not done
Case 7	1st biopsy (lymph node)	AITL-3	Positive	+
	2nd biopsy (lymph node)	EBV-CHL	Negative	+++# (Reed Sternberg cells)
Case 8	1st biopsy (lymph node)	AITL-3	Positive	+
	2nd biopsy (lymph node)	EBV-CHL	Negative	+++# (Reed Sternberg cells)
Case 9	1st biopsy (lymph node)	AITL-3	Positive	++
	2nd biopsy (lymph node)	AITL-3	Positive (poor fixation)	+++
	3rd biopsy (skin)	AITL*	Not interpretable	Not done
Case 10	1st biopsy (lymph node)	AITL (not reviewed)	Not done	N/A
	2nd biopsy (caecum)	AITL*	Positive	Not done
	3rd biopsy (tonsil)	AITL*	Positive	Not done
Case 11	1st biopsy (lymph node)	AITL-3 & EBV-DLBCL	Positive	+++
	2nd biopsy (lymph node)	AITL-2	Positive	Negative
Case 12	1st biopsy (lymph node)	AITL-3	Positive	+
	2nd biopsy (lymph node)	AITL-3	Positive	+++
Case 13	1st biopsy (lymph node)	AITL-3	Positive	++
	2nd biopsy (gastric)	PTL	Negative	Not done
Case 14	1st biopsy (lymph node)	AITL-2	Positive	Negative
	2nd biopsy (lymph node)	AITL-2	Positive	Negative
Case 15	1st biopsy (lymph node)	AITL-3	Positive	++
	2nd biopsy (lymph node)	AITL-3	Positive	++

Abbreviations: AITL-1,2,3, angioimmunoblastic T-cell lymphoma patterns I, II and III; *not assigned a "pattern" as involved site is extranodal; EBV, Epstein Barr virus; DLBCL, diffuse large B-cell lymphoma; PTL, peripheral T-cell lymphoma; EBER-ISH, Epstein Barr virus encoded RNA – in situ hybridization; N/A, not available, + to ++++, number of EBER positive cells ranging from a few scattered cells to a large number of positive cells. # LMP-1 positive; N/A, not available

Table 6.2 CD10 expression and EBER in situ hybridization

(continued from previous page)

	Biopsy	Diagnosis	CD10 positive T-cells	EBER-ISH
Case 16	1st biopsy (lymph node)	AITL-3	Not done	Not done
	2nd biopsy (lymph node)	AITL-3	Not done	Negative
Case 17	1st biopsy (lymph node)	AITL-3	Not done	Not done
	2nd biopsy (lymph node)	AITL-3	Not done	+++
	3rd biop;sy (skin)	AITL*	Not done	Negative
Case 18	1st biopsy (lymph node)	AITL-3	Not done	N/A
	2nd biopsy (lymph node)	EBV-DLBCL	Not done	Diffuse sheets of positive cells
	3rd biop;sy (lymph node)	AITL-3	Not done	N/A
Case 19	1st biopsy (lymph node)	AITL-1/2	Positive	N/A
	2nd biopsy (lymph node)	AITL-2	Positive	N/A
	3rd biopsy (lymph node)	AITL-3	Positive	N/A
	4th biopsy (lymph node)	AITL-1	Positive	N/A
Case 20	1st biopsy (lymph node)	AITL-3	Not done	N/A
	2nd biopsy (lymph node)	AITL-3	Positive	N/A
	3rd biopsy (skin)	AITL*	Not done	N/A
Case 21	1st biopsy (lymph node)	AITL-1	Positive	N/A
	2nd biopsy (lymph node)	AITL-2	Not done	N/A
	3rd biopsy (lymph node)	Not diagnostic	Not done	N/A
Case 22	1st biopsy (lymph node)	AITL-3	Positive	N/A
	2nd biopsy (skin)	AITL-3-	Not done	N/A
Case 23	1st biopsy (lymph node)	AITL-3	Not done	N/A
	2nd biopsy (skin)	AITL*	Not done	N/A
	3rd biopsy (oral)	AITL*	Not done	N/A
Case 24	1st biopsy (lymph node)	AITL-3	Negative	N/A
	2nd biopsy (lymph node)	AITL-2	Negative	N/A
Case 25	1st biopsy (lymph node)	AITL-3	Positive	N/A
	2nd biopsy (skin)	AITL*	Not done	N/A
	3rd biopsy (Waldeyer's ring)	AITL*	Not done	N/A
	4th biopsy (blood)	AITL*	Positive	N/A
Case 26	1st biopsy (lymph node)	AITL-3	Not done	N/A
	2nd biopsy (lymph node)	EBV-DLBCL	Negative	Diffuse sheets of positive cells
Case 27	1st biopsy (lymph node)	AITL-1	Positive	+
	2nd biopsy (lymph node)	AITL-3	Negative	++
Case 28	1st biopsy (lymph node)	AITL-3	Negative	N/A
	2nd biopsy (lymph node)	EBV negative DLBCL	Negative	Negative
Case 29	1st biopsy (lymph node)	AITL-3	Not done	Not done
	2nd biopsy (lymph node)	AITL-2	Not done	Not done

In case 18, a dominant B-cell clone could not be detected in the biopsy (showing AITL) that followed complete remission of the EBV-associated (monoclonal) DLBCL. In the same case however, the PCR results of the biopsy (showing AITL) that preceded the development of DLBCL were not available.

In one case (case 4) with EBV-associated DLBCL with involvement of the bone marrow, PCR was unsuccessful due to technical reasons.

6.2.4.2.2 *EBV-associated CHL*

A dominant B-cell clone could not be detected in both biopsies (cases 7 and 8) complicated by EBV-associated CHL.

6.2.4.2.3 *EBV-negative DLBCL*

The molecular genetics results were not available in case 28, where the biopsy at relapse showed features of an EBV-negative DLBCL

6.2.4.2.4. *A dominant B-cell clone in AITL, in the absence of features amounting to DLBCL on histology*

A dominant B-cell clone was detected in 3 cases (cases 6, 20 and 24) showing features of AITL, but no histological evidence of DLBCL. In one case (case 6), there were many scattered EBV-infected B-cells in the biopsy that harboured a detectable dominant clone and in the preceding (initial) biopsy where a dominant B-cell clone was not detected. In case 20, a dominant B-cell clone was detected in the initial biopsy and in the subsequent biopsies there were weak dominant bands, but size comparison of the bands was not possible in this case. In the 3rd case (case 24), a PCR result was available only on follow up where a dominant B-cell clone was detected.

6.2.5 Treatment given and survival data

These are summarized in Table 6.1. A variety of treatment regimens have been used to which there does not appear to be a predictable response. In the 18 patients where follow up clinical data was available, 11 patients died (duration of survival from initial diagnosis ranged from 11- 60 months [mean survival 26 months]), 5 were alive with disease (follow up, 12-247 months) and 2 cases (cases 8 and 28) were alive and well (follow up, 72 and 24 months respectively).

6.3 Discussion

6.3.1 Progression from pattern I (with hyperplastic follicles) to pattern II/III (typical AITL)

As 5 of the 6 cases showing pattern I histology on initial biopsy were misdiagnosed as reactive on initial biopsy, the histology in the consecutive biopsy represents natural histologic progression with no influence of treatment. Of these 5 cases, in the 3 cases that were investigated for CD10 expression, the neoplastic T-cells highlighted by CD10, not only increased in number, correlating well with the marked increase in FDC meshwork, but their distribution also changed from being in the vicinity of hyperplastic follicles in pattern 1 (figure 6.1K-N) to a more diffuse distribution with concentration around the expanded FDC meshworks (Figure 6.2I and J). The histologic progression from patterns I to III in consecutive biopsies is thus confirmation of the findings of Ree and co-workers (Ree *et al*, 1998), that those cases with pattern I histology, should be classified as AITL. The time interval between biopsies showing patterns I and II/III showed marked variation (2 – 168 months) which indicates that this may represent a

varied rate progression of the disease in different patients. When the interval is as short as 2 months (case 1) however, the possibility that this may represent varying degrees of involvement of different lymph nodes at one time in a particular patient also needs to be considered – i.e. limited involvement a lymph node in pattern I versus more extensive involvement in patterns II/III.

In case 19, the histology progressed from patterns I to II, and then to pattern III at 1st and 2nd relapse respectively. In this case, complete remission was achieved following treatment of each relapse (Table 6.1). However, at 3rd relapse, the histology showed "pattern I" histology on biopsy, indicating that this appearance may also be seen in relapse following successful treatment.

6.3.2 "Typical" AITL on initial biopsy and follow up lymph node biopsies

In 13 cases, the initial and follow up biopsies showed features of "typical AITL" which probably indicates extensive involvement of biopsied nodes at diagnosis and follow up. A lack of change in numbers or distribution of CD10 positive neoplastic T-cells in many of these cases, also suggests similar extent of lymph node involvement at diagnosis and follow up. In case 11 however, although there was no significant reduction in number, the CD10 positive tumour cells which showed a diffuse distribution in an effaced node (pattern III) at diagnosis, was more confined to a follicular distribution (pattern II) following treatment and probably indicates a favourable response to the latter.

6.3.3 "High grade" transformation

Prior to the era of immunohistochemistry, there are reports of progression of "AILD" to "immunoblastic lymphoma", with no reference to the lineage of the latter (Pangalis, et al 1983). Following the advent of immunohistochemistry, enabling lineage assignment, the reports of high-grade transformation have mainly been to B-cell "immunoblastic lymphoma" or DLBCL, many associated with EBV (Abruzzo *et al*, 1993;Bauer *et al*, 1982;Joly *et al*, 2004;Knecht *et al*, 1995;Matsue *et al*, 1998;Nathwani *et al*, 1978;Park *et al*, 2002;Pirker *et al*, 1986;Zettl *et al*, 2002).

The findings in our study confirm that in most instances of "high-grade" histologic "transformation" in AITL, the large cell lymphoma is EBV-associated and of B-cell rather than T-cell lineage. In the current study, in 3 cases (the initial biopsy in case 11 and the follow up biopsies in cases 6 and 18), the EBV-associated DLBCL comprised numerous EBV-infected large B-cells that co-existed with AITL in the same biopsy. Two other cases cases developed EBV-associated DLBCL with no evidence of AITL. One case (case 28) developed an EBV-negative DLBCL, an unusual feature to occur following complete remission of AITL diagnosed only 8 months previously. However, expansion of EBV-negative B-cell clones, although uncommon has been previously described (Brauninger *et al*, 2001). A single case (case 13) in the present study the follow-up biopsy showed a large cell lymphoma of T-cell lineage with no hint of any defining features of AITL. Although the latter could be clonally related to the preceding AITL, this was not proved by molecular genetics studies.

6.3.4 EBV-associated B-cell proliferations

EBV-infected lymphocytes have been reported in over 95% of cases in some studies.(Weiss *et al*, 1992) A variety of EBV-associated B-cell proliferations, have been reported in AITL. These are thought to occur due to the associated immune deficiency and include a prominent population of EBV-infected lymphoid cells, sometimes forming a confluent population in the background of AITL, while in others it has occurred as an EBV-associated DLBCL in patients with a previous history of AITL (Abruzzo *et al*, 1993;Knecht *et al*, 1995;Matsue *et al*, 1998;Park *et al*, 2002;Zettl *et al*, 2002). There are also few reports of the occurrence of CHL in a patient with a history of AITL (D'Arrigo *et al*, 1985;Melato *et al*, 1983;Nakamura *et al*, 1995). In the current study of 29 cases, 10 cases (34%) showed either an EBV-associated B-cell lymphoma or a dominant B-cell clone. Five cases were complicated by EBV-associated DLBCL and molecular genetics evidence of a dominant B-cell clone, while 2 cases developed EBV-associated CHL. In 3 other cases there was a dominant B-cell clone associated with a prominence of EBV-infected B-cells that did not amount to DLBCL.

6.3.4.1 DLBCL

The prognosis of AITL once complicated by EBV-associated DLBCL is not well known. In the study by Zettl and co-workers (2002), they report 2 cases of AITL that were complicated by EBV-associated DLBCL and 1 case complicated by EBV-associated plasmacytoma, 34-96 months from initial diagnosis. In the cases in the current study, the time taken to develop EBV-associated DLBCL varied from being present at initial diagnosis (case 11) to its development 84 months following initial diagnosis (case 26) suggesting varied susceptibility. In the 2 cases of EBV-associated

DLBCL reported in the study by Zettl, *et al* (2002) the clinical course varied, with one case dying from infection in 4 months, while the other patient had a complete remission of the DLBCL, but only to relapse with DLBCL 10 months later and die of infection 3 months later. The case in their series that was complicated by EBV-associated plasmacytoma was alive with disease 24 months after diagnosis of plasmacytoma. In our study, 2 of the cases (cases 5 and 6), the patients died within 3 months of diagnosis of EBV-associated DLBCL. However, in 2 other cases (cases 11 and 18) complete regression of the DLBCL was achieved. In case 11, the complete regression of EBV-infected B-cells was achieved following treatment with thalidomide. In case 18, the patient received multi-agent chemotherapy. This suggests that the prognosis following development of EBV-associated DLBCL is quite varied and may also depend on what treatment the patients receive. Recently, GELA (France) has recognized the need to combat the B-cell component in AITL and has commenced a phase II trial using Rituximab with CHOP for AITL with a prominent B-cell component. Similarly, in the UK, a trial is being launched using Fludarabine and cyclophosphamide with the addition of thalidomide which has been included not only for its anti-angiogenic properties but also in order to investigate its value in controlling the expansion of EBV-infected B-cells (Dogan *et al*, 2005); personal communication).

6.3.4.2 Classical Hodgkin lymphoma (CHL)

Nakamura, *et al* (1995) reported the occurrence of EBV-associated CHL with no evidence of T-cell lymphoma, 16-years following AITL that had no EBER positivity at initial diagnosis. In our series we have 2 cases that developed EBV-associated CHL 5-years following AITL. As EBV-infected Reed-Sternberg-like cells and a polymorphous

background are regular features of AITL, the diagnosis of CHL in a patient with a history of AITL is a difficult one. This is especially so, as the CD30 positive Reed-Sternberg-like cells in AITL may also be CD15 positive (Quintanilla-Martinez *et al*, 1999). In fact a diagnosis of CHL can be made with any degree of confidence only in the absence of AITL. The latter is difficult to prove on morphology alone as the absence of cytological features of malignancy does not rule out AITL, but the absence of FDC and vascular proliferation and lack of molecular genetics evidence of a T-cell lymphoma helps to exclude it.

6.3.5 Concluding remarks

AITL with hyperplastic follicles represents an early form of AITL, an appearance that may also on occasion be seen at relapse following complete remission. When AITL is complicated by the occurrence of a "large cell lymphoma" it is most often an EBV-associated DLBCL. However a PTL with large cell morphology or EBV-negative DLBCL may also occur. EBV-associated B-cell lymphomas such as DLBCL and less commonly CHL may occur in up to 25% of cases of AITL.

Chapter 7

CELL OF ORIGIN OF ANGIOIMMUNOBLASTIC T-CELL LYMPHOMA

7.1 Introduction

In B-cell lymphomas, characterization of normal cell counter part of specific neoplasms has greatly enhanced our ability to classify and risk stratify. In contrast in T-cell lymphomas no such scheme is in place. If present it would help better classification and risk stratification.

In AITL, FDC proliferation is one of the defining histological features (Jaffe & Ralfkiaer, 2001a). In chapter 3, we showed that the CD3+, CD4+, CD8- neoplastic T-cells in AITL also express CD10 and show a distribution closely associated with that of the expanded FDC meshwork. It was also shown that in early cases with hyperplastic follicles and minimal FDC expansion, the CD10+ neoplastic cells are seen in the outer rim and vicinity of hyperplastic follicles. In peripheral lymphoid tissues, CD10 expression appears to be associated with the GC microenvironment, and is restricted to GC B-cells (Arber & Weiss, 1997) and the more recently documented, minor subset of benign CD10+ T-cells located mainly within GCs (Cook *et al*, 2003). All these features raise the possibility that the neoplastic T-cells in AITL may be derived from GC T-cells. The latter form a distinct subset of T-cells that are confined to the GC and express CD4 and CD57. A small proportion of these cells also express BCL-6. In a recent study production of B-cell chemokine (BLC), also known as B-cell chemo-attractant (BCA) or

CXCL13 has been shown to be a feature unique to GC T-cells, setting it apart from other T-cells (Kim *et al*, 2004).

The investigation of the exact phenotype and cytokine secretion profile of the neoplastic T-cell in AITL, a pre-requisite to identifying the cell of origin, is complicated by the fact that the neoplastic T-cells are invariably outnumbered by a prominent reactive component that also includes numerous reactive T-cells and a varied number of "atypical" EBV-infected bystander B-cells (Anagnostopoulos *et al*, 1992;Khan *et al*, 1993;Willenbrock *et al*, 2005).

Therefore, the aim of this study was to use CD10 as a marker of the neoplastic T-cell in AITL and investigate whether the latter expresses the GC- T-cell antigens, CD57 and BCL-6, and the chemokine CXCL13.

7.2 Results

7.2.1 Tissues used in this part of the project

Forty five paraffin embedded lymph node biopsies from 45 cases of AITL, diagnosed on clinical, histologic, immunophenotypic and molecular genetic criteria were reviewed and the diagnosis confirmed in each case. These forty-five (biopsies) cases, all of which showed CD10 expression by the neoplastic T-cells, were selected to evaluate whether the CD10+ neoplastic T-cells expressed CD57 (26 cases) and BCL-6 (20 cases) and CXCL13 (22 cases).

7.2.2 Histology

The architecture of all lymph nodes examined was either partially or completely effaced by a polymorphic infiltrate comprising, small lymphocytes and transformed blasts, plasma cells, histiocytes and eosinophils within a prominent vascular network.

Depending on the presence of hyperplastic or regressed follicles or the absence of follicles, the histology could be categorized into the previously described patterns I, II and III.

Table 7.1: Histology and percentages of CD57+ T-cells and CD10+ T-cells, and BCL-6 expression CD10+ tumour cells.

Case no	Pattern	Clear cells	CD57+ T-cells/ CD3+ T-cells	CD10+ T-cells/ CD3+ T-cells	BCL-6 expression by tumour cells
1	3	Present	10%	20%	Variably positive
2	3	Absent	10%	50%	Variably positive
3	3	Present	10%	40%	Not done
4	3	Absent	≤5%	50%	Variably positive
5	3	Present	≤5%	40%	Variably positive
6	3	Absent	30%*	30%**	Variably positive
7	3	Present	20%	50%	Variably positive
8	3	Present	30%	50%	Not done
9	3	Absent	10%	20%	Variably positive
10	3	Present	10%	60%	Variably positive
11	3	Absent	20%	50%	Variably positive
12	3	Absent	≤5%	15%	Variably positive
13	3	Present	20%*	30%**	Variably positive
14	3	Absent	20%	40%	Variably positive
15	3	Absent	30%	20%	Variably positive
16	3	Absent	50%*	80%**	Variably positive
17	2	Present	10%	30%	Variably positive
18	2	Absent	10%	20%	Not done
19	2	Present	≤5%	20-30%	Variably positive
20	2	Absent	≤5%	40%	Variably positive
21	2	Absent	10%	15-20%	Variably positive
22	2	Absent	≤5%	15-20%	Not done
23	1	Present	≤5%	20%	Not done
24	1	Absent	10%	20%	Variably positive
25	1	Absent	10%	20%	Not done
26	1	Absent	20%	15-20%	Variably positive

*Medium or medium to large CD57+ T-cells
** A subpopulation of CD10+ T-cells appear to co-express CD10 and CD57

Abbreviations: +, positive

7.2.3 Immunohistochemistry

7.2.3.1 CD57 is not expressed by the neoplastic T-cell in most cases of AITL

Serial sections stained with CD57, CD3, CD10, CD20 and CD21 were examined to estimate the approximate ratio of CD57 positive cells, and CD10 positive neoplastic T-

cells, to all (CD3 positive) T-cells, and assess the distribution of CD57 positive cells in relation to CD10 positive neoplastic T-cells, and CD21 FDC meshworks. In selected cases, the distribution of CD57 positive cells was also assessed using CD10/CD57 and CD57/CD21 double-layered immunohistochemistry. These results are given in Table 7.1. In 23 of 26 cases, CD57 staining highlighted small cells, distinct from the CD10+ neoplastic T-cell population (Figure 7.1A-F). Of these cases, in those with typical morphology (18 cases), the distribution of CD57+ cells showed no definite association with the FDCs. In those showing "pattern 1" histology with hyperplastic GCs (5 cases), the CD57+ T-cells showed a diffuse, interfollicular distribution with concentration within some follicles. In the remaining 3 cases (with typical morphology) the CD57 cells were mainly medium-sized or medium to large, somewhat atypical lymphoid cells and showed a diffuse distribution with concentration around expanded FDCs (Figure 7.1J and L). In these latter 3 cases, the distribution of CD57+ cells showed an overlap with CD10+ cells and double staining showed a subpopulation that appeared to co-express CD10 and CD57 (Figure 7.1K)

7.2.3.2 BCL-6 is expressed by the neoplastic T-cell in AITL

In all cases examined BCL-6 was expressed although in varied intensity, by cells that showed a similar distribution to CD10+ T-cells (Table 7.1M). When assessing the latter, residual CD10+ (GC) B-cells were excluded by comparing sequential sections stained with CD20 and CD10 and by double-layered CD20/CD10 immunohistochemistry. In cases where assessment was difficult owing to varied staining intensity of BCL-6 expression in individual cells, the pattern of expression was clarified by CD10/BCL-6 double-staining (Figure 7.1N and O). In the 7 cases that showed clear cells, a variable pattern of BCL-6 expression was observed in the clusters of pale to clear tumour cells.

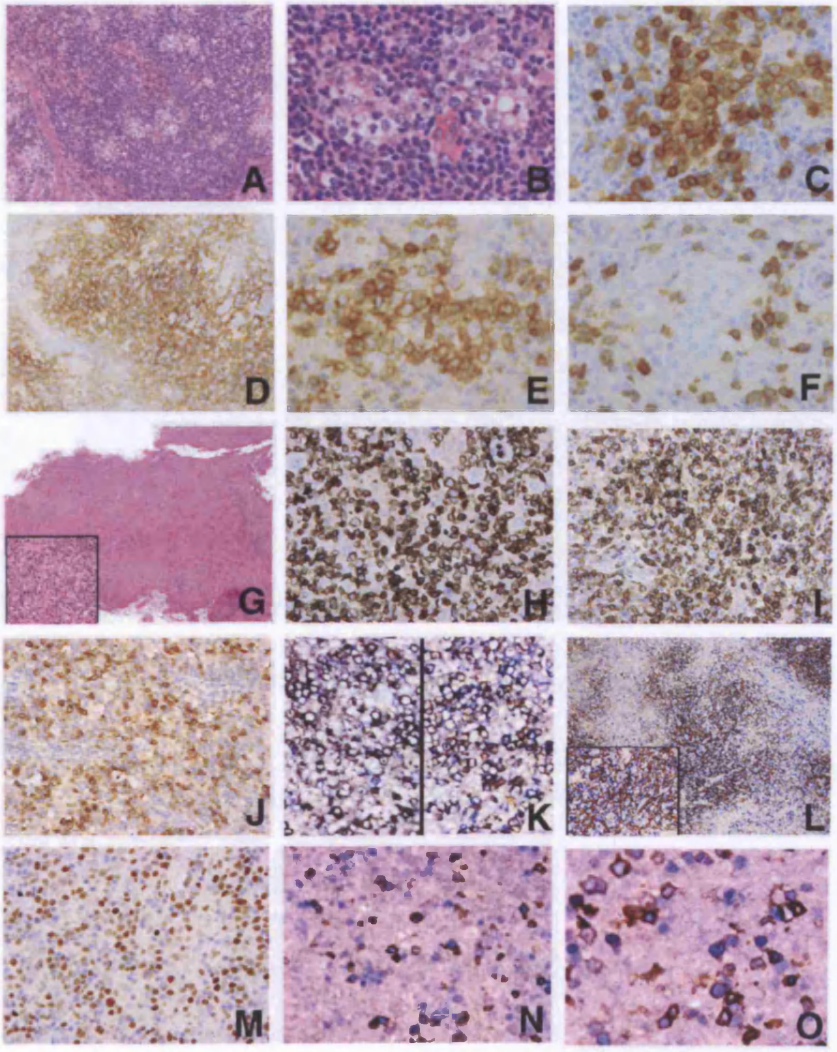

Figure 7.1 CD57 and BCL-6 expression in AITL

Panels A-F show biopsy from case 23. (A and B) Haematoxylin and eosin (H&E) stained section showing clusters of clear cells. (C) CD3 stain confirms that the clear cells are T-cells. (D) CD21 showing mild expansion of the follicular dendritic cell (FDC) meshwork. (E) CD10 stain highlights clusters of clear cells. (F) CD57 stain shows small CD57 positive T-cells but the clear cells are CD57 negative. Panels G-M show biopsy from case 13. (G) H&E stained section showing a "pattern III" histology with clear cells in a polymorphous background (inset). (H) CD3 stain highlights numerous T-cells. (I) CD10 stain shows many CD10 positive neoplastic T-cells. (J) CD57 highlights many CD57 positive cells. (K, left panel) CD10/CD57 double stain and (K, right panel) CD57/CD10 shows many lymphoid cells that appear to co-express CD10 and CD57. (L) CD21/CD57 double stain shows that the CD57 positive cells are concentrated around the CD21 positive expanded FDC (M) BCL-6 stain highlights many positive cells. Panels N and O show CD10/BCL6 double staining in another case (case 2) shows many CD10 (brown) positive cells show variable co-expression of BCL6.

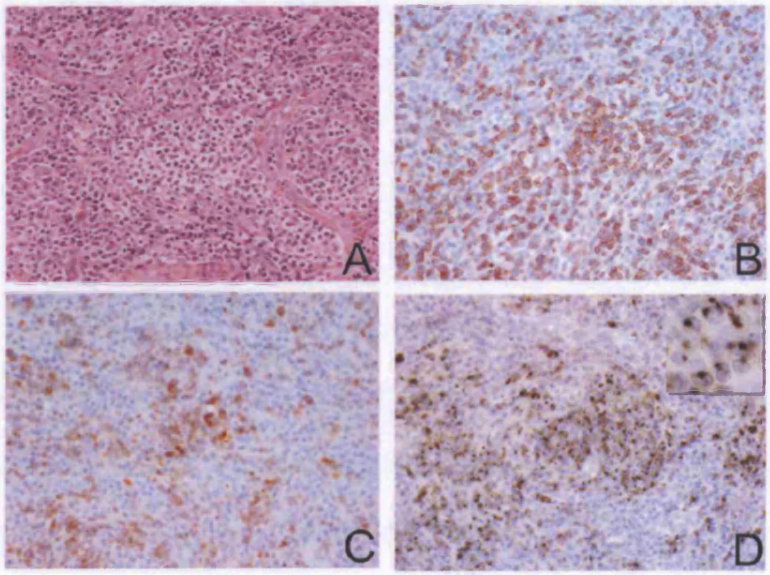

Figure 7.2 CXCL-13 expression by the neoplastic T-cells in AITL.

(A) Haematoxylin and eosin stained section showing clusters of clear cells. (B) CD3 highlights these clusters of clear cells. (C) CD10 stain shows that the neoplastic T-cells (clear cells) are CD10 positive. (D) CXCL-13 stain shows positive staining in the clear cells (neoplastic T-cells).

7.2.3.3 CXCL13 is expressed by the neoplastic cell in AITL

Similarly, CXCL13 expression by tumour cells was assessed by single-layered immunohistochemistry. All 22 cases of AITL (where the neoplastic cells were CD10 positive) showed CXCL13 positivity. In all cases examined, the distribution of CXCL13 positivity was identical to the distribution of CD10+ tumour cells (Figure 7.2).

7.2.4 Microdissection and PCR for TCRγ gene rearrangement: in a subset of cases, part of the neoplastic T-cell population expresses CD57

As 3 cases showed a rather large subpopulation that appeared to co-express CD10 and CD57, in one such case (case 16 – Table 7.1), CD57+ cells were microdissected by laser capture (Figure 7.3) and subject to PCR for TCRγ gene rearrangement. PCR products from microdissected cells gave a band identical in size to the dominant band observed for the PCR performed on whole sections (Figure 7.4), suggesting that in these cases, at least part of the neoplastic population expressed CD57.

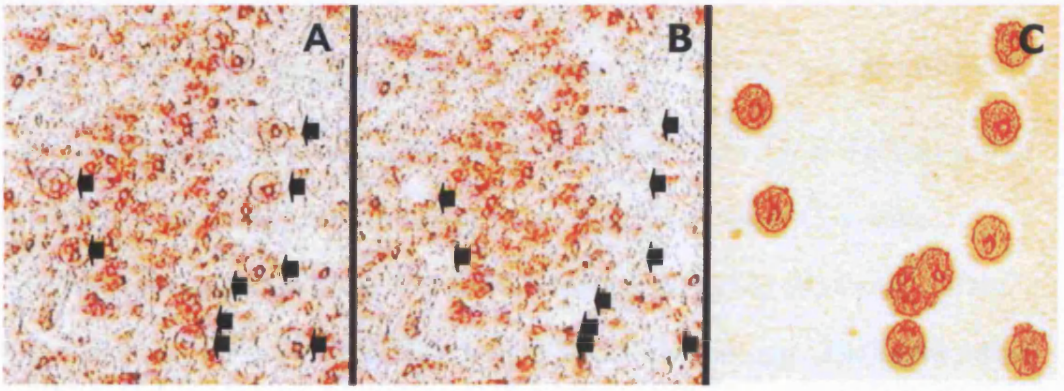

Figure 7.3. Laser capture microdissection of CD57 positive lymphoid cells in AITL.

(A) CD57 stained section before microdissection; the cells targeted for microdissection are indicated within the black arrows. (B) Same area after microdissection; the black arrows show the spaces left after cells have been removed. (C) High-power view of a cluster of CD57 positive cells after microdissection. These are the cells/spaces indicated by the arrows in panels A and B. Original magnification A, B, 3 200; C 3 400.

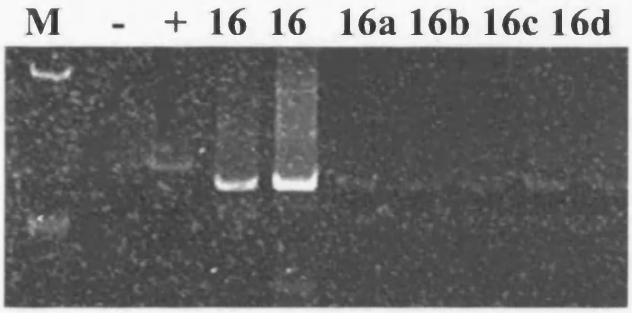

Figure 7.4. Case 16: analysis of PCR products for TCR-γ chain gene (from whole section and microdissected samples) on a 10% polyacrylamide gel.

Lane 1 (M), molecular weight markers; lane 2, negative control; lane 3, positive control; lane 4and 5 whole section PCR products from case 16; lanes 6-9 (16a-e) microdissected PCR products from case 16.

7.3 Discussion

7.3.1 Germinal centre (GC) T-cells

GC T-cells comprise a distinct subset of CD4+ effector T-cells that is localized to GCs and efficient at inducing B-cell antibody production. In addition to CD4, these lymphocytes also express CD2, CD3 and more specifically CD57 (Kim *et al*, 2001b). GC T-cells also express the activation marker CD69 (Schaerli *et al*, 2000) and a subset has been shown to express BCL-6 (Falini *et al*, 1996;Flenghi *et al*, 1995). Recently Cook, and co-workers (2003) reported the presence of a minor population of benign CD10 positive T-cells that are located mainly within GCs. GC-T-cells proliferate poorly in response to T-cell activation (Johansson-Lindbom *et al*, 2003), and are CD45RA- and CD45RO+ (Kim *et al*, 2001b), features that would be consistent with terminal differentiation. On activation, GC-T-cells express inducible antigens CD134 (OX40) and CD40L (Kim *et al*, 2001b), which enable interaction with GC-B-cells (Calderhead *et al*,

1993;Stuber *et al*, 1995), and also express high levels of interleukin-10 (IL-10) (Kim *et al*, 2001b), a cytokine important for antibody class switching and B-cell differentiation into plasma cells (Liu & Banchereau, 1997). Furthermore, inducible co-stimulator (ICOS) and cytotoxic T-lymphocyte-associated molecule-4 (CTLA-4), members of the CD28 family of co-stimulatory molecules are highly expressed on GC-T-cells (Breitfeld *et al*, 2000;Vandenborre *et al*, 1998). ICOS, when activated, superinduces the synthesis of IL-10 (Hutloff *et al*, 1999). Although it was believed that GC-T-cells were unable to secrete IL-2, TNF-α and IFNγ (Bowen *et al*, 1991;Velardi *et al*, 1986), more recent reports (Kim *et al*, 2001b), show that they are able to produce IL-2, IL-4, IFNγ and TNF-α. Their chemokine receptor phenotype of expressing CXCR5 (the receptor for BCA/CXCL13), but not CCR7 [the receptor for EB11-ligand chemokine (ELC)] correlates well with their localization to GC's, as they chemotax to B-cell chemo-attractant - CXCL13, but not T-cell zone chemokine – ELC) (Breitfeld *et al*, 2000;Kim *et al*, 2001b;Schaerli *et al*, 2000).

More recently, gene expression profiling has shown that CXCL13 is one of the most highly up-regulated genes in GC-T-cells. The latter, but not other T-cells, secrete large amounts of functional CXCL13 upon T-cell receptor activation (Kim *et al*, 2004).

7.3.2 CD57 and its expression in AITL

The CD57 antigen is a 110-kd glycoprotein that is encoded by a gene on chromosome 11 (Schroder *et al*, 1983). In peripheral blood it is expressed by NK-cells and a subset of T-cells that is predominantly of the CD8+ cytotoxic phenotype (Abo & Balch, 1981;Arber & Weiss, 1995;Phillips & Lanier, 1986). In lymphoid tissues CD57+ T-cells are CD3+, CD4+, CD8-, located primarily in the GCs and are referred to as GC-T-cells (Poppema

et al, 1983;Si & Whiteside, 1983;Swerdlow & Murray, 1984;Velardi *et al*, 1986). The structure and function of CD57, associated molecules and its relevance to disease is not known. The most common CD57+ lympho-proliferative disorder is T-cell large granular lymphocytic leukaemia (T-LGL) (WHO, 2001). CD57 is not usually expressed by extranodal NK/T-cell lymphoma, nasal type or aggressive NK-cell leukaemia (Chan *et al*, 2001a;Chan *et al*, 2001b). Although most haematopoietic neoplasms do not express CD57, it may be expressed by a wide range of non-haematopoietic neoplasms such as prostatic adenocarcinomas, thyroid carcinomas and oligodendrogliomas, among others (Ghali *et al*, 1992;Liu *et al*, 2004;Loy *et al*, 1994;Motoi *et al*, 1985). Following most bone marrow and some solid organ transplantation and in HIV infection, there is a well-documented increase in CD8+, CD57+ T-cells (Leroy *et al*, 1986;Mizuno *et al*, 1986;Reipert *et al*, 1992) and a few reports of an increase in CD4+, CD57+ T-cells (Legendre *et al*, 1989;Velardi *et al*, 1988) in the peripheral blood. Non-neoplastic reactive CD57+ GC-T-cells are often increased in number in nodular lymphocyte predominant Hodgkin lymphoma (NLPHL), where they form rosettes around the neoplastic L&H cells (Kamel *et al*, 1993;Poppema, 1989).

With regard to AITL, Ree and co-workers (1999) reported that in "AITL with GCs" (3 cases), many GCs were devoid of CD57+ cells while others were loosely populated with CD57+ cells and scattered CD57+ cells were also seen in the interfollicular areas, suggesting outward migration of intra-follicular T-cells. Their results with double staining for CD57 and BCL-6 showed that approximately 90% of CD57+ cells were BCL-6 negative, the staining being equivocal in the remaining 10% suggesting that they are 2 distinct populations. Although Ree and co-workers (1999) describe the distribution of CD57+ T-cells in early AITL, the status of the neoplastic T-cell with

regard to CD57 expression is not entirely clear from their study. In the present study, all cases showed a varied number of CD57+ T-cells (<5%-50%). With the aid of CD10 as a marker of neoplastic T-cells and the use of both single and double layered immunohistochemistry, our results show that in most cases, the CD57 T-cell population represents small T-cells, forming a distinct population from CD10 positive T-cells including atypical "clear" cells when present, suggesting that the former are probably reactive in nature (Figure 6.1A-F.). These findings are consistent with those inferred in the study by Ree and co-workers*l* (1999). However in 3 of 26 cases, the CD57+ cells, were diffusely distributed but with some concentration around the expanded FDC meshwork, and included medium sized or medium to large and sometimes clearly atypical cells. In these cases double staining showed that a subpopulation of the CD10+ neoplastic T-cells appeared to co-express CD57 (Figure 6.1K). Preliminary molecular genetics analysis of TCRγ gene rearrangement in one of the latter cases showed that the size of the PCR product obtained from micro-dissected samples was identical to that obtained from the whole section. These results suggest that the CD57+ population in this case form at least part of the neoplastic clone. However, as interpretation (and comparison) of band size on a 10% polyacrylamide gel is not entirely accurate and an identical band size does not always mean identical sequence, because the difference in DNA sequence may result from a difference in (N-region) composition, rather than number of base pairs, confirmation of these findings would require DNA sequence analysis. Overall, in most cases of AITL, CD57+ T-cells are probably reactive but in a minority of cases, a subset of the neoplastic population appears to co-express CD57.

7.3.3 BCL-6 and its expression in AITL

BCL-6, a nuclear phosphoprotein, with transcriptional repressor activities, is expressed by GC-B-cells and critical for GC development. It is induced in B-cells during GC induction and down-regulated on GC cell differentiation. BCL-6 is also expressed by a small subset of CD57+ GC-T-cells (Falini *et al*, 1996), and a small fraction of interfollicular CD4+ T-cells (Flenghi *et al*, 1995). On expression profiling, Kim and co-workers (2004) and Chtanova and co-workers (2004) have shown that BCL-6 is up-regulated in GC-T-cells. The function of BCL-6 in T-cells is not yet known. Kraus and Haley (Kraus & Haley, 2000) showed that the reactive CD57+ T-cells that rosette the neoplastic L&H cells in NLPHL, also co-express BCL-6.

The results of the present study show that the tumour cells in AITL express BCL-6 in varying intensity, a feature confirmed by the use of double-layered immunohistochemistry in selected cases demonstrating co-expression of CD10 (membrane stain) and BCL-6 (nuclear stain) in T-cells, including clusters of "atypical clear cells". These findings are in concordance with those of Ree and co-workers (1999) who reported a similar pattern of BCL-6 expression in "atypical tumour cells" in 10 cases of AITL.

7.3.4 CXCL-13 and its expression in AITL

CXCL-13, the ligand for CXCR5 plays a crucial role in attracting B-cells to follicles containing FDCs (Ansel *et al*, 2000). CXCL13 is believed to be expressed by FDCs, and as mentioned above has been shown to be a characteristic of GC-T-cells, setting them apart from other T-cells (Kim *et al*, 2004). It has also been shown to be expressed by dendritic cells and endothelial cells of HEV (Ebisuno *et al*, 2003;Vissers *et al*, 2001).

Many of the CXCL13+ GC-T-cells are in direct contact with antigen presenting CD11C+ cells and GC-B-cells (Kim *et al*, 2004). Freshly isolated GC-T-cells secrete low levels of CXCL13, in the absence of stimulation. T-cell activation, similar to T-cell receptor activation increases CXCL13 expression by 20-40 fold (Kim *et al*, 2004). Anti-CD3 in combination with anti-CD28, and also interaction with GC-B-cells stimulate CXCL13 production by GC-T-cells (Kim *et al*, 2004). In-vitro studies have shown that CXCL13 in GC-T-cells efficiently attracts CD4+ memory T-cells, GC-T-cells and IgD+ naïve B-cells (Kim *et al*, 2004).

Ohshima and co-workers (Ohshima *et al*, 2004) reported CXCL13 expression in AITL. Using CD10 as a marker of the neoplastic T-cell we showed that the latter co-expresses CXCL13, consistent with the chemokine secretion profile of GC-T-cells. One of the downstream effects of CXCL13 expression is induction and proliferation of FDCs probably via stimulation of lymphotoxin alpha production by B-cells (Ansel *et al*, 2000). In this light, it would be interesting to investigate whether CXCL13 plays a role in the FDC proliferation in AITL, a feature considered to be a morphological hallmark of the disease

7.3.5 Cell of origin in AITL – Concluding remarks

The neoplastic T-cell in AITL is known to be of the CD3+, CD4+ phenotype and in chapter 3 we showed that it also expresses CD10. In the present study using CD10 as a marker for the neoplastic T-cell we show that it expresses BCL-6, and in a minority of cases, CD57. As chemokine receptors have been used as markers of functional subsets of T-cells, the CXCR3+, CD134+ (OX40), CD69+ and T-box transcription factor (T-

bet)+ profile in AITL has hitherto been interpreted as evidence of Th1 differentiation (Dorfman & Shahsafaei, 2002;Dorfman *et al*, 2003;Ishida *et al*, 2004;Jones *et al*, 1999;Jones *et al*, 2000;Ohshima *et al*, 2004;Tsuchiya *et al*, 2004). However, GC-T-cells, which have only recently been identified as a subset that is distinct from Th1 and Th2 effector subtypes, are also known to express CD134 (OX40) and the activation marker CD69 (Kim *et al*, 2004). Furthermore, the results of our study have shown that the neoplastic T-cells express CXCL13, a marker that sets GC-T-cells apart from other T-cells. Despite the lack of CD57 expression in most cases, overall, the features favour a GC-T-cell origin for the neoplastic T-cell in AITL. Whether they arise from a specific subset of GC-T-cells is however, yet to be determined.

Chapter 8

CORRELATION BETWEEN EBV LOAD AND HISTOLOGY, AND THE ROLE OF HHV-8 IN ANGIOIMMUNOBLASTIC T-CELL LYMPHOMA

8.1 Introduction

AITL is characterised by prominent systemic symptoms and has been shown to be associated with a state of immune dysregulation and the production of a variety of cytokines (Foss *et al*, 1995;Pautier *et al*, 1999;Pizzolo *et al*, 1990;Siegert *et al*, 1992;Siegert *et al*, 1995;Takeshita *et al*, 1993;Tobinai *et al*, 1988). These features are similar to those of conditions such as multicentric Castleman disease that have a proven viral (HHV-8) aetiology in many cases (Dupin *et al*, 2000).

Although it is widely accepted that AITL is indeed a T-cell lymphoma, there is an ongoing debate as to whether an oligoclonal process precedes the emergence of lymphoma. (Jaffe & Ralfkiaer, 2001a) This suggests that the disease may be associated with the effects of an agent that induces T-cell proliferation, which in turn would give rise to a dominant clone possessing genetic changes that confer growth advantage. A possible viral aetiology has been entertained and EBV has been the most extensively investigated as a potential causative agent. Although some studies (Anagnostopoulos *et al*, 1992;Anagnostopoulos *et al*, 1995) have suggested that T-cells may also be infected, the overall consensus is that the EBV infection does not involve T-cells but t an expansion of EBV infected B-cells may be present in up to 95% of cases (Brauninger *et*

al, 2001;Ohshima *et al*, 1994;Weiss *et al*, 1992). The latter is believed to be a secondary phenomenon due to the immune dysregulation present.

Other viruses such as HHV-6, (Daibata *et al*, 1997;Luppi *et al*, 1993) HHV-8 (Luppi *et al*, 1996) and hepatitis C virus (Luppi & Torelli, 1996), have also been reported in AITL. HHV-6 although identified by PCR, (Luppi *et al*, 1993) was localized to scattered by-stander plasma cells (Luppi *et al*, 1998) by immunohistochemistry. There have also been conflicting reports regarding the presence of HHV-8 infection in AITL (Chadburn *et al*, 1997;Luppi *et al*, 1996).

In chapter 3 we showed that histological pattern I represents limited lymph node involvement, while pattern III shows extensive lymph node involvement by the disease. Furthermore in chapter 6 we showed histological progression from pattern I to patterns II/III in sequential biopsies.

The aim of this part of the project was two-fold. The first was to characterise and immunophenotype the cell-type of EBV-associated cell proliferations in AITL and to correlate the EBV load with the histological patterns (I-III) previously described.

In the second part of this study we investigated for the presence of HHV-8 virus in AITL.

8.2 Results

8.2.1 Tissues used in this part of the study

Paraffin embedded biopsies from 63 diagnosed cases of AITL were reviewed and the histology confirmed in each case. Paraffin embedded tissue was available in all cases, and frozen material was available on 5 cases.

8.2.1.1 Characterisation of EBV-infected cells

Four cases (Cases 1, 2, 40 and 41) selected for this part of the study showed numerous EBV-positive cells on EBER in situ hybridisation and had histological features of DLBCL (lymph node biopsy at relapse in case 1, and bone marrow biopsy in case 41) and/or evidence of a dominant B-cell clone on Ig heavy chain gene PCR (lymph node biopsies in cases 2 and 40).

8.2.1.2 EBV quantification by real time PCR

Paraffin embedded tissues from 49 biopsies (39 cases [Cases 1-39]) were used for this part of the study. Frozen material available in 5 of the cases was also utilised.

8.2.1.3 HHV-8 screening by conventional PCR

Paraffin embedded tissues from 28 cases of AITL (Cases 1, 11, 12, 19, 29, 36 and 42-63), were used for this part of the study.

8.2.2 Characterisation of EBV-infected cells in AITL

8.2.2.1 Double layered immunohistochemistry and EBER in situ hybridisation: EBER positive cells are CD79a positive

Four biopsies from 3 cases (with many EBER positive cells) were selected for double layered immunohistochemistry and EBER in situ hybridisation. One case (case 1) had consecutive biopsies (case 5 in chapter 6) the first of which had many EBER positive cells but no evidence of a dominant B-cell clone on IgH PCR, and the second which had numerous confluent sheets of EBER positive cells and a dominant B-cell clone,

amounting to DLBCL. The other 2 cases (cases 2 and 40) had many scattered EBER positive cells and a dominant B-cell clone. The combinations of double layered immunohistochemistry and EBER-ISH were as follows: CD3/EBER, CD20/EBER, CD79a/EBER and CD10/EBER. The EBER positive population was distinct from the CD3 and CD10 positive populations in all 4 biopsies (Figures 8.1). On CD20/EBER, although some EBER positive cells were CD20 positive, there were many cells that were CD20 negative (Figure 8.2). However, CD79a highlighted all EBER positive cells (Figure 8.2).

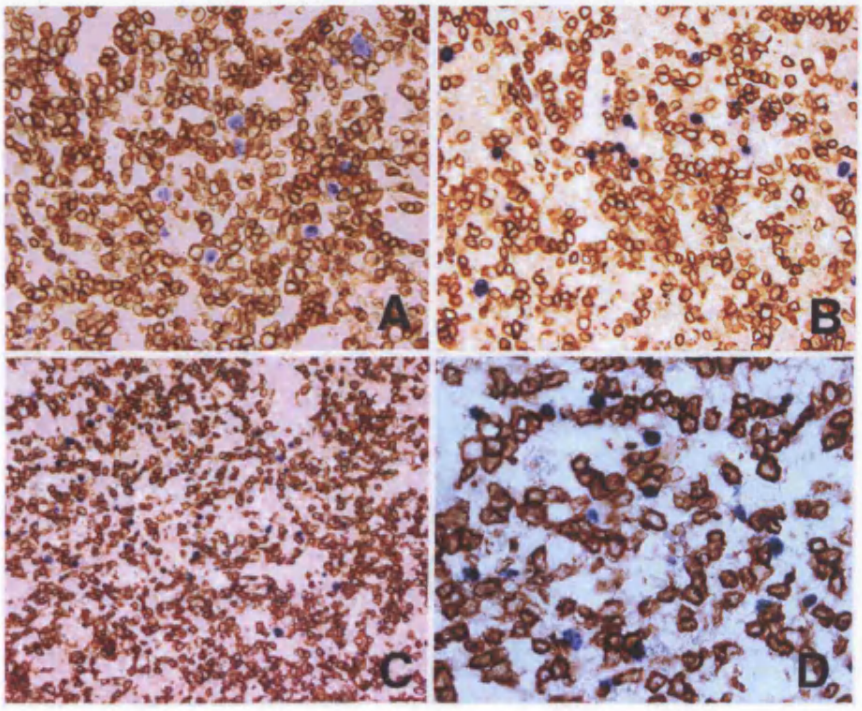

Figure 8.1. EBER positive cells are CD3 negative

EBER-in situ hybridization/CD3 staining in case 2 (panel A), case 1 (panel B) and case 40 (panels C and D) show that the EBER positive population (blue) is distinct from the CD3 positive T-cells (brown)

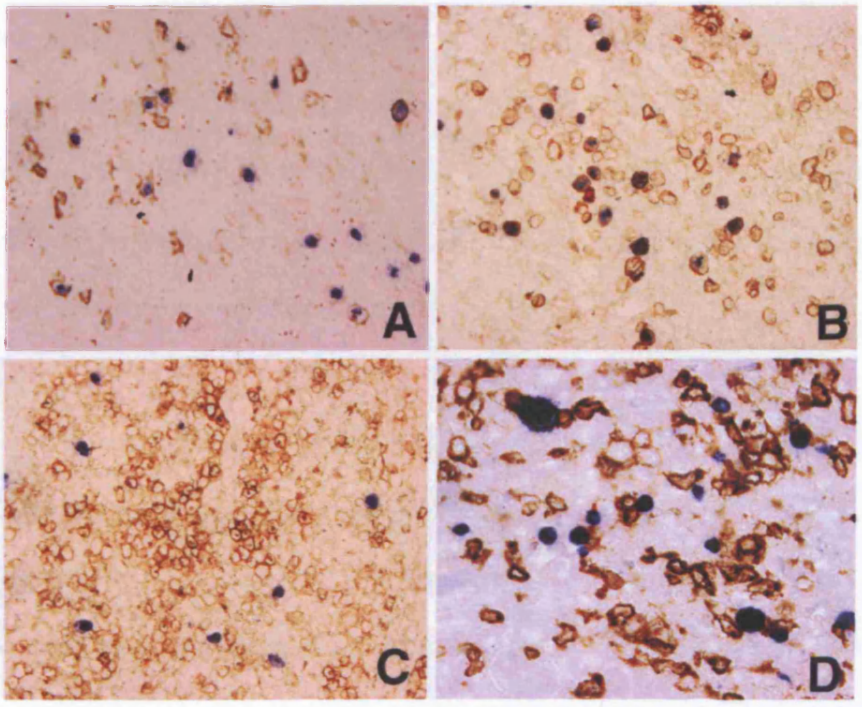

Figure 8.2 EBER positive cells are CD20 +/-, CD79 positive and CD10 negative.

(A) EBER/CD20 in case 2 shows that only some of the EBER positive cells express CD20. (B) EBER/CD79a in case 2 highlights all EBER positive cells. (C and D) EBER/CD10 in case 2 (C) and case 40 (D) shows that the EBER positive population is distinct from the CD10 positive cells.

8.2.2.2 Further immunophenotypic characterisation of EBER positive B-cells: EBV-infected B-cells in AITL show an immunoblastic / plasmacytoid phenotype

For further immunophenotypic characterisation, sections stained using single layered immunohistochemistry were compared with consecutive sections on which EBER-ISH

was performed. When examining sections, areas where EBER positive cells were present in sheets were selected for comparison.

BCL-6 performed on cases 1 and 41 showed that the EBER-ISH positive cells were BCL-6 negative (Figure 8.3). MUM-1/IR4 and CD138 immunostaining performed on case 41, showed that the EBER-ISH positive cells were MUM-1/IR4 positive (Figure 8.3), but CD138 negative. Immunoglobulin light chain staining in case 40 showed specific staining with abundant cytoplasmic Ig and and kappa light chain restriction in the EBV positive B-cell population (Figure 8.4)

8.2.2.3 Microdissection of EBER positive cells and IgH PCR: EBV-infected B-cells comprise the dominant B-cell clone (when present) in AITL

EBER-ISH positive cells from case 40 were microdissected by laser capture and IgH PCR was performed on single microdissected cells. The band size in 2 of the 4-microdissected samples was identical to the dominant band on whole section PCR (Figure 8.4) confirming (the immunohistochemical observation of light chain restriction) that EBV-infected cells comprise the dominant clone.

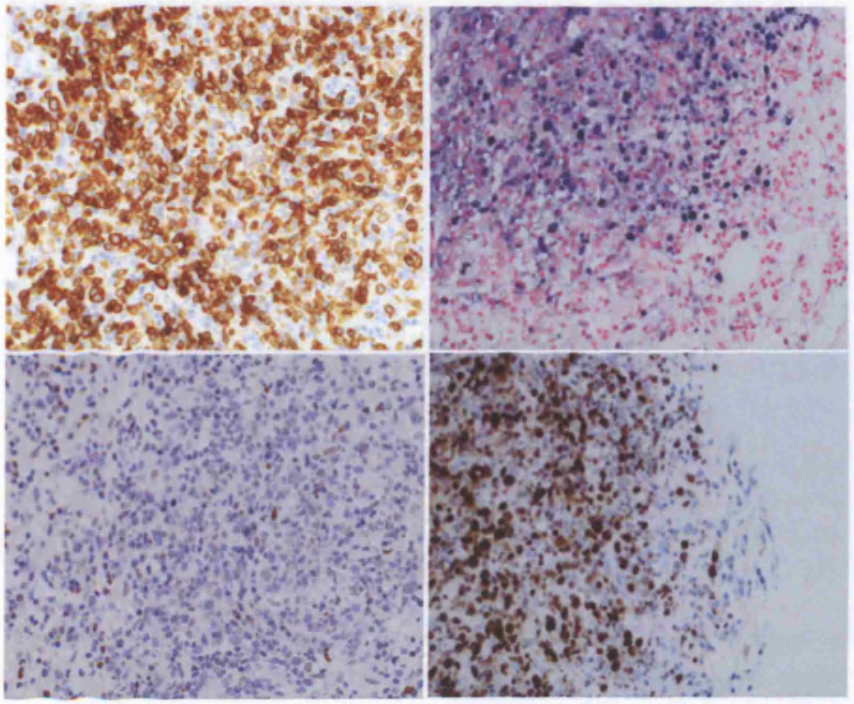

Figure 8.3. EBER positive cells in EBV-associated diffuse large B-cell lymphoma in bone marrow of case 41, are CD79a positive, BCL6 negative and MUM-1 positive.

(A) CD79a highlights numerous B-cells. (B) EBER-in situ hybridization shows numerous EBV infected cells in same area. (C and D) Deeper section showing same area, with cells that are negative for BCL-6, but positive for MUM-1.

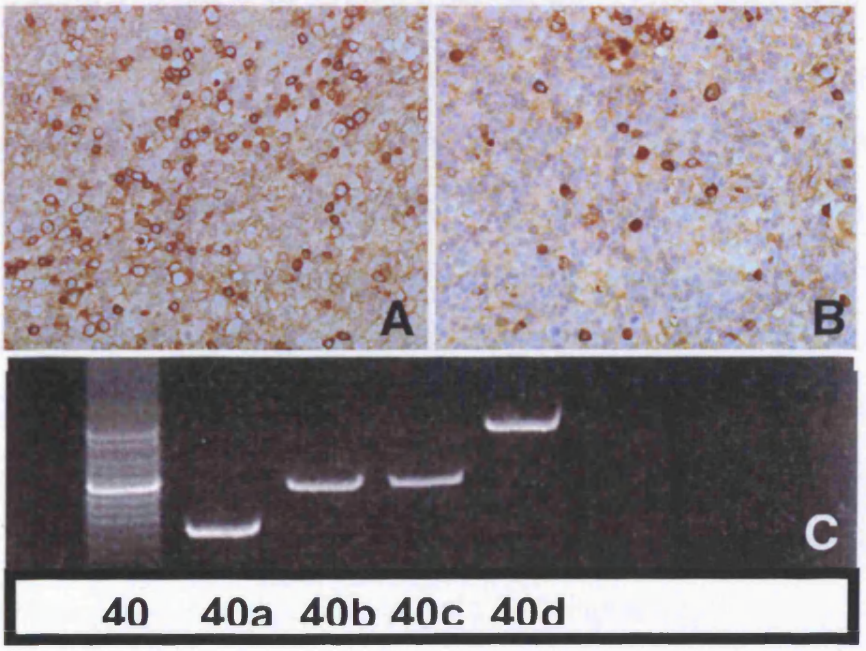

Figure 8.4 Light chain immunostaining and immunoglobulin heavy chain gene PCR of whole section and microdissected EBER-positive cells in case 40 show that EBER positive cells comprise dominant clone.

(A and B) Kappa (A) and lambda (B) immunostaining shows kappa light chain restriction. Lambda staining highlights a few scattered plasma cells only.
(C) PCR products of whole section (lane 1) and microdissected EBER positive cells (lanes 2-5) show that the product size of the microdissected samples in lanes 3 and 4 are identical in size to that of the whole section dominant band.

8.2.3 Virus specific EBV quantitative real-time PCR

Forty-nine biopsies from 39 cases (Table 8.1) were selected for quantitative PCR using EBV-specific primer pairs designed from EBNA region for EBV (106bp), the results of which are shown in Table 8.1 and Figure 8.4. For details of method of quantification see Chapter 2, section 2.5.9.3.

8.2.3.1 A high EBV load, a feature predominantly of pattern III histology

Real-time, quantitative PCR using EBV virus-specific primers was successful in 48 biopsies (39 cases) (Table 8.1). Of these, 24/27 biopsies (22/25 cases), showing pattern III histology were EBV positive (Tables 8.1). Of the 27 "pattern III" biopsies, 15 biopsies had a high EBV load of >50 copies/1000 cells (Table 8.1 and Figure 8.4). Of the 13 biopsies showing pattern II histology, 10 were EBV positive by PCR, but only 2 biopsies showed an EBV load of >50 copies/1000 cells (Table 8.1 and Figure 8.4). Three of the 4 biopsies showing pattern I histology were EBV positive, but none of them had a high EBV load (>50 copies/1000 cells) (Table 8.1 and Figure 8.1).

8.2.3.2 Cases with consecutive biopsies

The results of EBV-virus specific quantitative PCR, in cases where consecutive biopsies were available for study, are summarised in Table 8.1

8.2.3.2.1 *Progression from "Pattern I" to "Pattern III" associated with marked rise in EBV load*

In the 2 cases which showed histologic progression from patterns I to III (cases 32 and 33), the initial biopsies with pattern I histology was either negative or had a low EBV load, whereas the consecutive biopsies had a high EBV load (> 50 copies/1000 cells).

8.2.3.2.2 *Consecutive biopsies with "Pattern III" histology show a decrease or mild ncrease in EBV load in follow up biopsy*

Of the 3 cases where consecutive biopsies showed "pattern III" histology (cases 34-36), one case (case 34) had a high EBV load in both biopsies, but the first biopsy had a much

higher load than the second biopsy. The involved parotid biopsy that followed was also EBV-positive, but with a lower viral load than in previous lymph node biopsies. In case 35, only the follow up biopsy had a successful result and was negative for EBV, while in case 36, both biopsies were EBV positive, with a viral load of <50 copies/1000 cells.

8.2.3.2.3 *Regression of EBV-associated lympho-proliferation in AITL Pattern III and DLBCL treated with Thalidomide*

In case 37 (case 11 in chapter 6, treated with Thalidomide between biopsies), the initial biopsy which showed AITL and co-existent EBV-DLBCL had a very high EBV load, a feature that had regressed in the follow up, post-treatment biopsy.

8.2.3.2.4 *Absence of EBV in PTL that followed EBV positive AITL*

In case 38 although EBV was present in the first biopsy showing AITL, this was not the case in the consecutive biopsy showing diffuse sheets of large atypical T-cells, classified as PTL, unspecified.

8.2.3.2.5 *High EBV load in a case of AITL, that developed CHL 5-years later*

In case 39, where the patient developed EBV-associated CHL 5-years following AITL, a high EBV load was detected in the initial biopsy showing AITL.

8.2.4 HHV-8, virus specific PCR: HHV-8 infection is not a feature of AITL

All 28 cases investigated were negative for HHV-8, virus-specific PCR

Table 8.1 Summary of results of real-time EBV -specific PCR

	Site of biopsy	Diagnosis	Pattern	EBV copies/1000 cells (triplicate, mean)
1	Lymph node	AITL	3	71.3
2	Lymph node	AITL	3	53.4
3	Lymph node	AITL	3	7.5
4	Lymph node	AITL	3	18.4
5	Lymph node	AITL	3	156.1
6	Lymph node	AITL	3	10.4
7	Lymph node	AITL	3	3.5
8	Lymph node	AITL	3	54.2
9	Lymph node	AITL	3	3462.6
10	Lymph node	AITL	3	359.1
11	Lymph node	AITL	3	86.4
12	Lymph node	AITL	3	232.3
13	Lymph node	AITL	3	499.1
14	Lymph node	AITL	3	0
15	Lymph node	AITL	3	21.8
16	Lymph node	AITL	3	0
17	Lymph node	AITL	3	14.4
18	Lymph node	AITL	2	0
19	Lymph node	AITL	2	94.9
20	Lymph node	AITL	2	25.2
21	Lymph node	AITL	2	0.9
22	Lymph node	AITL	2	0
23	Lymph node	AITL	2	47.6
24	Lymph node	AITL	2	7.7
25	Lymph node	AITL	2	8.9
26	Lymph node	AITL	2	36.4
27	Lymph node	AITL	2	0
28	Lymph node	AITL	2	6.5
29	Lymph node	AITL	2	512.6
30	Lymph node	AITL	1	12.4
31	Lymph node	AITL	1	3.4

Table 8.1 Summary of results of real-time EBV -specific PCR (continued from previous page)

	Site of biopsy	Diagnosis	Pattern	EBV copies/1000cells (triplicate, mean)
32	Lymph node	AITL	1	14.7
	Lymph node	AITL	3	55.7
33	Lung	AITL	1	0
	Lymph node	AITL	3	1124.4
34	Lymph node	AITL	3	418.5
	Lymph node	AITL	3	65.3
	Parotid gland	AITL		19
35	Lymph node	AITL	3	failed
	Lymph node	AITL	3	negative
36	Lymph node	AITL	3	15.4
	Lymph node	AITL	3	35.4
37	Lymph node	AITL +EBV- DLBCL	3	3697.9
	Lymph node	AITL	2	2.4
38	Lymph node	AITL	3	33.9
	Lymph node	PTL, unspecified	N/A	0
39	Lymph node	AITL	3	291.3
	Lymph node	EBV-CHL	N/A	13.6

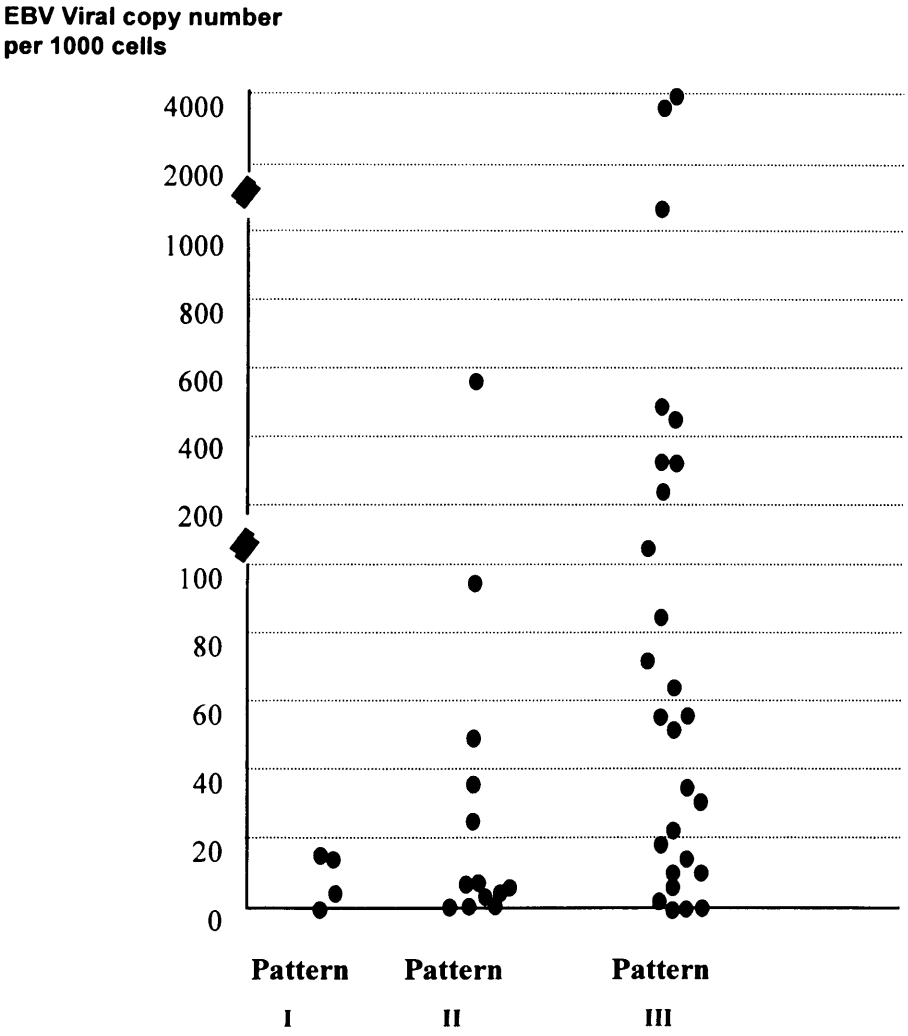

Figure 8.5: Correlation between EBV load and histological pattern of AITL in tumour tissues

8.3 Discussion

8.3.1 EBV infection in B-cells and its occurrence in AITL

EBV, a γ herpes virus with a double stranded DNA genome, infects mainly B-cells, but may also infect T-cells and epithelial cells (Kuppers, 2003). EBV infection usually occurs in childhood or adolescence when it results in an asymptomatic or mild illness (Cohen, 2003;Kuppers, 2003), following which the virus remains in a latent state for life (Yao *et al*, 1985). In individuals with impaired cellular immunity, virus reactivation occurs (Babcock *et al*, 1999), while in the immunosuppressed, an uncontrolled EBV-driven B-cell proliferation may occur (Ho *et al*, 1988;Shapiro *et al*, 1988).

In healthy virus carriers EBV infects naïve B-cells, which proliferate and undergo clonal expansion. Some of these B-cells then undergo the GC reaction and differentiate into memory B-cells (Thorley-Lawson, 2001). EBV remains latent in these memory cells, but if the latter differentiate into plasma cells, the virus may switch to a lytic cycle (Laichalk & Thorley-Lawson, 2005) and infect new naïve B-cells, thus maintaining the pool of virus-infected cells (Babcock *et al*, 2000;Joseph *et al*, 2000a;Joseph *et al*, 2000b;Thorley-Lawson & Babcock, 1999;Thorley-Lawson, 2001). Although many studies support this model for infection in healthy carriers, in vitro experiments suggest that EBV may not just target naïve B-cells, but may also infect memory B-cells (Ehlin-Henriksson *et al*, 2003). There is also some speculation as to whether the GC characteristics acquired by EBV-infected B-cells occurs in "extrafollicular" regions (Thorley-Lawson, 2001).

In infectious mononucleosis, EBV infects GC/memory B-cells and also naïve B-cells (Kurth *et al*, 2000). Kurth, *et al* showed that clonal expansion in infectious mononucleosis occurs mainly in B-cells with mutated Ig genes that show no intraclonal diversity.

EBV-associated post-transplant lympho-proliferative disorder (PTLD) which in most instances is of B-cell origin, may be polyclonal, oligoclonal or monoclonal (Harris *et al*, 2001b). The majority however is monoclonal and most clones have mutated Ig genes, a fraction of which show on-going somatic hypermutations (Brauninger *et al*, 2003). The occurrence of "crippling mutations" in Ig genes and survival without selection for expression of a functional antigen receptor have also been reported, suggesting that EBV may interfere with normal B cell differentiation and selection processes in PTLD (Brauninger, *et al* 2003).

In AITL, EBV-infection has been reported mainly in bystander B-cells, but a few studies report infection in T-cells. Reports in the literature (Anagnostopoulos *et al*, 1992;Anagnostopoulos *et al*, 1995) demonstrating EBV infection of T-cells in AITL used CD45RO to identify T-cells. However as transformed B-cells and pre-plasma cells may also be reactive with anti-CD45RO (Hathcock *et al*, 1992;Jensen *et al*, 1989), reactivity to the latter is not proof of T-cell lineage. Most studies (Brauninger *et al*, 2001;Ohshima *et al*, 1994;Weiss *et al*, 1992) including the present study, have shown that EBV infection is confined to B-cells. As in PTLD, expansion of EBV-infected B-cell clone in AITL is thought to be a result of immune deregulation.

Brauninger and co-workders (2001) showed that most EBV infected B-cells in AITL carry mutated Ig genes, indicating a memory or GC-like genotype. They also showed that the subset of EBV infected B-cells resembling memory B-cells showed little

tendency for clonal expansion, whereas the proliferating EBV infected B-cells undergoing clonal expansion showed evidence of on-going somatic hypermutations, indicating a GC-like genotype. They go on to postulate that in AITL, the large aggregates of FDCs, in association with the CD4 positive T-cells, may simulate their role in normal GCs and provide the required microenvironment to induce or maintain somatic hypermutations in some of these cells. Furthermore as described for PTLD (Brauninger *et al*, 2003), Brauninger, *et al* (2001) demonstrated that many of the expanding B-cell clones had acquired destructive mutations in originally functional V gene rearrangements, over repeated rounds of mutation and division, showing that somatic mutations are acquired without selection for expression of a functional antigen receptor. Although seen mainly as a feature of EBV infected B-cells, it was also occasionally observed in EBV negative B-cells in AITL (Brauninger *et al*, 2001).

8.3.2 EBV-infected B-cells in AITL have an immunoblastic / plasmacytoid immunophenotype

Several studies (Brauninger *et al*, 2001;Ohshima *et al*, 1994;Weiss *et al*, 1992) used double labelled immunohistochemistry / in situ hybridisation and showed that most EBER positive cells co-expressed CD20. In our study double labelling showed that some EBER positive cells express CD20 but all seem to be highlighted by CD79a. In contrast, the EBER positive cells were distinct from the CD3 and CD10 positive lymphoid populations (Figure 8.3). The MUM-1 positive, CD10 negative, BCL-6 negative, CD79a positive, CD138 negative, CD20 +/- immunophenotype together with the presence of abundant cytoplasmic light chain Ig, point to an immunoblastic/plasmacytoid phenotype. This phenotype is similar to that observed in

EBV-driven PTLD with a "post-GC" phenotype (Capello *et al*, 2003). In the light of the findings by Brauninger, *et al* (2001), the "post-GC", rather than a "GC" phenotype (in EBV-infected cells) is unexpected, as all examples selected to investigate the EBV-infected B-cell phenotype in the present study had numerous EBER-ISH positive cells amounting to DLBCL and/or a dominant B-cell clone on IgH PCR, indicative of clonal expansion. Whether some of the genotypic characteristics such as acquisition of GC-like features with ongoing somatic mutations, and survival despite "crippling" mutations are the result of the viral infection and the association with the tumour/FDC environment is yet to be determined.

8.3.3 Histologic progression from patterns I to III, and its correlation with EBV load

In chapter 3, 3 overlapping histologic patterns ("patterns I-III") were described in AITL. They showed increasing destruction of the normal lymph node architecture from patterns I through to III, and were defined by the presence of hyperplastic follicles (pattern I), regressed follicles (pattern II) or absence of identifiable follicles (pattern III). The appearance described as "pattern I" histology has also been described by Ree, *et al* (1998) as "AITL with hyperplastic follicles", and shown (in 2 cases) to progress to more "typical AITL" suggesting that "pattern I" may represent the histological appearance in "early" AITL. This was confirmed in the results presented in chapter 6 where 4 cases showing "pattern I" morphology progressed to more typical AITL ("patterns II/III") on subsequent biopsy. Furthermore, in chapter 3, it was shown that the number and distribution of CD10 positive neoplastic T-cells in patterns I, II and III, were consistent with histologic progression, as they increased in number from patterns I-III and

progressed from being confined to the region of the follicles (pattern I) to a more diffuse distribution correlating with the increasing expansion of FDCs (pattern III). AITL is characterised by immune deregulation resulting in susceptibility to opportunistic pathogens, which would result in re-activation of latent viral infections. A high EBV-load was mainly a feature in biopsies with "pattern III" histology. Furthermore, in the two cases where sequential biopsies showed progression from "Pattern I to "Pattern III", there was a dramatic rise in EBV load at second biopsy. The correlation of EBV load with the histological pattern, being more prevalent in cases with pattern III histology, may be related to the increasing immune-deficiency in more advanced disease. As AITL is characterised by a prominent reactive infiltrate, these may contribute the histology observed as "pattern III". However, the occurrence of this pattern in the absence of a high EBV load indicates that these are not essential or do not play a significant role in defining the pathology of "pattern III".

8.3.4 HHV-8 infection in AITL

HHV8 or Kaposi sarcoma herpesvirus (KSHV), a γ herpes virus, the aetiological agent in Kaposi sarcoma, is also causatively linked with lympho-proliferative conditions such as multicentric Castleman disease and the plasmablastic lymphoma that it may give rise to, HHV-8 and EBV associated germinotropic lymphoproliferative disorder and primary effusion lymphoma, the latter most often found in the setting of HIV infection.
On morphology, the prominence of regressed follicles in some cases of AITL shows an overlap with the histology of HHV-8 associated Castleman disease (Frizzera, 2001). AITL, like multicentric Castleman disease is associated with immune dysregulation and is characterized by prominent systemic symptoms, which are attributed to cytokine

production. Thus the clear overlap between clinical and pathological features of the two conditions warrants investigation of HHV8 as a possible aetiological agent in AITL. In support of this, Luppi, *et al* (1996) reported detection of HHV8 sequences in 3 cases of AITL by PCR. However, Chadburn *et al*, (1997) failed to observe any evidence of HHV8 infection by PCR or immunohistochemistry. In the present study, using virus-specific PCR we were unable to detect evidence of HHV8 in 28 cases of AITL excluding the likelihood of a causative role.

There are a few reports in older literature showing an association of "AILD" with Kaposi sarcoma (Friedman-Birnbaum *et al*, 1985;Kluin-Nelemans *et al*, 1984;Suster *et al*, 1987). Considering the morphologic overlap between the two conditions, and paucity of adjuncts such as immunohistochemistry and molecular diagnostics available for diagnosis at the time of these studies, these cases probably represent examples of HHV-8 related Castleman disease rather than AITL.

8.3.5 Concluding remarks

In AITL, EBV infected B-cells in AITL show an immunoblastic/plasmacytoid immunophenotype.

EBV load correlates well with histological patterns I-III described in chapter 3. A high EBV load is mainly a feature of "pattern III" histology, and probably parallels the increasing immunosuppression in these patients.

HHV8 infection is not a feature of AITL.

Chapter 9

OVERVIEW

9.1 CD10 is expressed by the neoplastic T-cells in AITL and is a sensitive and specific marker of the disease.

In chapter 3, CD10 was investigated as a possible phenotypic marker of AITL. As the neoplastic cells comprise the minority and are vastly outnumbered by reactive cells, it was necessary to microdissect individual CD10 positive lymphoid cells for molecular genetic analysis. The results showed that the neoplastic T-cells in AITL expressed CD10, thus providing a marker to identify the neoplastic T-cell. In chapter 4, it was shown that in the assessment of nodal PTLs, CD10 expression appears to be specific to AITL. CD10 was thus shown to be a sensitive (85%) and specific (100%) marker of the disease. As CD10 immunostaining can be performed in any histopathology laboratory, this provides a very useful diagnostic marker and a useful adjunct to diagnosis. Furthermore, identification of a specific marker for the neoplastic T-cells in AITL, for the first time provides the opportunity to investigate the biology of the disease and may be in the future, devise novel therapeutic approaches.

9.2 The (CD10 positive) neoplastic T-cells in AITL account for a minority of total T-cells in most cases, and only a small proportion of them are in cycle

In chapter 3 it was shown that CD10 positive tumour cells accounted for only 10-30% of T-cells in most cases, confirming the studies of others that the neoplastic cells are in the minority. Although the overall proliferation fraction as assessed by single layered Ki 67 immunostaining, was high, double-layered immunohistochemistry showed that the majority of CD10 positive T-cells were Ki 67 negative. This raised the question whether CD10 expression in neoplastic T-cells in AITL may be an indicator of disturbed apoptotic cell death analogous to follicular lymphoma, and whether AITL is a biologically indolent/low grade tumour causing disease and death by immune deregulation rather than increased tumour load?

9.3 CD10 expression is maintained at most extranodal sites and correlates with the presence of FDCs

In chapter 5, we studied CD10 expression in extranodal sites involved by AITL. CD10 expression was maintained in most extranodal sites of involvement, except bone marrow. CD10 expression at extranodal sites correlated well with the presence of FDCs, suggesting an analogy with follicular lymphoma, where CD10 expression is down regulated in bone marrow and interfollicular areas lacking FDCs.

9.4 AITL has 3 overlapping histologic patterns

In chapter 3 it was also shown, that based on histology, 3 overlapping histological patterns could be identified, depending on the presence of hyperplastic (pattern I) or regressed (pattern II) follicles or the complete absence of identifiable follicles (pattern III). The FDC meshwork showed minimal/ no expansion in pattern I, but was hyperplastic in patterns II and III, with marked expansion in pattern III. An increase in CD10 positive T-cells was observed from patterns I to III. Furthermore, the distribution of the CD10 expressing neoplastic T-cells was perifollicular in pattern I and diffuse in pattern III, but concentrated around the markedly hyperplastic FDC meshwork.

9.4.1 Pattern I represents an early phase of AITL

In chapters 3,4 and 6 it was shown why AITL, pattern I should be classified as AITL, rather than PTLu. In chapter 6 we showed histological progression from pattern I to III, confirming earlier reports that that the appearance we describe as pattern I represents early lymph node involvement by AITL.

9.4.2 Partial phenotype is more consistent with AITL

In chapter 4 it was shown that the category described as AITL/PTL indeterminate had morphologic features of pattern III AITL such as a polymorphous infiltrate with or without clear cells and prominent vascularity but lacked the prominent FDC hyperplasia characteristic of AITL. However there was subtle FDC expansion with a tendency to encircle vessels. CD10 was also expressed by neoplastic T-cells in the majority. AITL/PTL indeterminate cases, also showed evidence of EBV infection in a high percentage of cases, a feature seen in AITL but not observed in any PTLu studied.

Furthermore, on molecular genetic analysis, the pattern of T-cell clonality in these cases was similar to AITL, with a dominant clone/clones being identified in a very high proportion (88%) of cases. Overall the evidence suggests that these cases are best regarded as AITL rather than PTLu.

9.4.3 Do we need to revise existing diagnostic criteria?

The findings in chapter 4 showed that the existing pathological criteria apply only to the "typical" histology that probably accounts for approximately 80% of all cases. This calls to question whether AITL is underdiagnosed and whether we need to revise existing criteria.

9.5 EBV-associated B-cell proliferation is a frequent complication of AITL

In chapter 6 we studied the histology of sequential biopsies. It was noted that when AITL is complicated by the occurrence of a "large cell lymphoma" it is most often an EBV-associated DLBCL. However a PTL with large cell morphology or EBV-negative DLBCL may also occur. EBV-associated B-cell lymphomas such as DLBCL and less commonly CHL may occur in up to 25% of cases of AITL. Evidence in one case showed that Thalidomide maybe useful in treating EBV-associated B-cell proliferations in AITL.

9.6 AITL originates from germinal center T-cells

In chapter 7 we used CD10 as a phenotypic marker and show that the neoplastic T-cells in AITL express BCL-6 and CXCL13. Although the neoplastic T-cells in most cases were CD57 negative this phenotypic profile favours a GC T-cell origin.

9.7 EBV infected B-cells have an immunoblastic/plasmacytoid phenotype

In chapter 8 we showed that the immunophenotype of EBV infected B-cells (in cases showing a dominant B-cell clone) were CD79a positive, CD20 +/-, BCL6 negative, CD10 negative and MUM-1 positive with abundant cytoplasmic Ig, consistent with an immunoblastic/plasmacytoid phenotype.

9.8 EBV-load correlates well with histological pattern of AITL

In chapter 8 we showed that a high EBV load is mainly a feature of "pattern III" histology, possibly due to increasing levels of immunosuppression that parallel histologic progression.

9.9 HHV-8 infection is not a feature of AITL

Finally in chapter 8, by PCR, we show that HHV-8 infection is not associated with AITL.

9.10 Diagnostic applications, research investigations derived from this study and the direction of future research activity

Following the publication of results included in this thesis (Attygalle *et al*, 2002;Attygalle *et al*, 2004). Lee and colleagues (Lee *et al*, 2003a) and Bassegio and co-workers (Baseggio *et al*, 2004) confirmed our findings by using flow cytometry, and showed CD10 expression in AITL in involved lymph nodes, extranodal sites and blood.

As CD10 immunostaining can be performed in any histopathology laboratory and flow cytometry performed in many haematology/haematopathology laboratories, CD10 expression by the neoplastic T-cells in AITL, provides a very useful phenotypic marker and a valuable adjunct to diagnosis, even at an early stage of evolution of the disease. Furthermore, the presence of such a marker provides an opportunity to establish more objective criteria for diagnosis than those currently in use.

As the neoplastic T-cell in AITL are in the minority, CD10 expression provides a means of identifying the tumour cells for phenotypic, genetic and functional studies:

Cytokine profile: Immunohistochemistry, in situ hybridisation and gene expression profiling may be used to investigate the cytokine secretion profile of the neoplastic T-cells.

Chromosomal and cytogenetic changes: Previous studies describing cytogenetic (clonal and clonally unrelated) abnormalities used whole tissue that contained not only neoplastic T- cells, but also include varying numbers of EBV-infected B-cells/ proliferations (Schlegelberger *et al*, 1994b). Microdissection of CD10 positive tumour cells would limit the investigation to tumour cells alone. CGH and the more recently described comparative expressed sequence hybridisation (CESH) could be performed on microdissected material and double labelled fluorescence studies (CD10/FISH) performed to investigate chromosomal and cytogenetic abnormalities in the neoplastic T-cells.

Apoptosis and AITL: The possible correlation between CD10 expression and apoptosis has been discussed in chapter 3, sections 3.3.3 and 3.3.4. Following the publication of our results (Attygalle *et al*, 2002), Kim, et al showed that the FDCs and endothelial cells in AITL express Fas ligand (FasL) whereas the CD10 expressing tumour T-cells express

Fas (CD95) and caspase 3, indicating Fas-FasL interaction between the neoplastic T-cell and the FDC and suggest that the follicular milieu is necessary for CD10 expression by tumour cells, a feature that may play a functional role in regulating apoptosis (Kim *et al*, 2002a).

Functional studies: Establishment of CD10 positive lymphoma cell lines and the study of the effects of pharmacological inhibitors of CD10 (neutral endopeptidase) activity, which may provide an opportunity to investigate the biology of the disease and in the future, to devise novel therapeutic approaches in the management of AITL.

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CD10 Expression in Extranodal Dissemination of Angioimmunoblastic T-cell Lymphoma

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Abstract: Angioimmunoblastic T-cell lymphoma (AITL) is a systemic disease that often has evidence of extranodal involvement at presentation. In a recent study of lymph nodes in AITL, we showed that the neoplastic T cells in most cases can be identified by aberrant expression of CD10. The aim of this study was to investigate whether CD10 expression by the neoplastic T cells is maintained in extranodal sites. Ten cases of AITL with histologic and immunophenotypic evidence of extranodal dissemination were studied. Seven cases of peripheral T-cell lymphoma unspecified (PTLu), that included biopsies of involved extranodal sites, two cases of enteropathy type T-cell lymphoma (ETTL), and one case of extranodal NK/T lymphoma, nasal type were selected as controls. Diagnostic lymph node biopsies and biopsies of extranodal sites were reviewed. PCR for T-cell clonality and single layer immunostaining for CD3, CD20, CD10, and CD21 and double layer immunostaining for CD20/CD10 were performed. All 10 cases of AITL had characteristic histologic features and molecular evidence of the disease in lymph node biopsies. In these cases, aberrant CD10 expression was maintained in the lung, cecum, tonsil, nasopharynx, and one of six involved bone marrow trephines. In these extranodal biopsies, the distribution of CD10-positive tumor cells correlated with that of the follicular dendritic cell meshwork (FDC). The five bone marrow trephines that lacked aberrant CD10 expression were devoid of morphologic and immunohistochemical evidence of FDC. In these five cases, there was evidence of aberrant CD10 expression in other involved sites that had FDC. The neoplastic cells in PTLu, ETTL, and extranodal NK/T lymphoma, nasal type were CD10 negative. Our data show that aberrant CD10 expression is a useful phenotypic marker for diagnosis of AITL in most involved extranodal sites, except bone marrow, and suggest a possible role of FDC in the pathogenesis of AITL.

Key Words: angioimmunoblastic, peripheral T-cell lymphoma, extranodal, CD10

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Angioimmunoblastic T-cell lymphoma (AITL) is a nodal peripheral T-cell lymphoma characterized by systemic disease and prominent constitutional symptoms.[1,9,21,22] Although generalized lymphadenopathy is a prominent feature, clinical evidence of extranodal involvement is often present at diagnosis. This includes hepatosplenomegaly seen in 50% to 70%, skin rash in 50%, pleuropulmonary involvement in 40%, and bone marrow involvement in 60% to 80% of patients[3,9,10,18,21,22,25] The almost universal occurrence of lymphadenopathy permits a diagnosis based on examination of a lymph node biopsy and extranodal sites, other than bone marrow are rarely subjected to histologic examination. Occasionally, an extranodal site is biopsied either as a diagnostic procedure or to examine the extent of tumor involvement and rule out infectious or inflammatory conditions. In these situations, diagnosis can be very challenging as the conventional criteria based on alterations in lymph node biopsies are not applicable. Histologically, involved lymph nodes show partial or total obliteration of the normal architecture by a polymorphic infiltrate of lymphocytes, plasma cells and eosinophils, prominent proliferation of venules, and expansion of the follicular dendritic cell (FDC) meshwork.[4,8,11] Collections of cells with pale to clear cytoplasm, described as being typical for AITL, is not a consistent finding and in many instances cytologic features of malignancy are not readily identifiable.[1] Therefore, despite histologic criteria, definite diagnosis is often difficult even on lymph node biopsy, leading to an error in initial diagnosis in >50% of the cases, further complicating histologic interpretation of an extranodal biopsy.[1]

In a recent study of lymph nodes in AITL, we showed that the neoplastic T cells in most cases can be identified by aberrant expression of CD10, a feature absent in reactive lymphoid proliferations and other peripheral T-cell lymphomas.[1] Early and accurate diagnosis of AITL in lymph nodes can thus be achieved by immunostaining for CD10. Aberrant CD10 expression, if maintained at extranodal sites of involvement, would serve as a phenotypic marker and a very useful diagnostic tool. The aim of the present study was to investigate whether the expression of CD10 by neoplastic T cells is maintained in extranodal sites.

MATERIALS AND METHODS

Tissues

Seventy-eight cases of AITL, diagnosed on lymph node biopsy, on clinical, histologic, immunophenotypic and molecular genetic criteria, were retrieved from the archives of the Department of Histopathology, University College London Hospital. Of these, 10 cases that included biopsies of involved extranodal sites were selected for study. All had been referred from other institutes. Seven cases of nodal peripheral T-cell lymphoma, unspecified (PTLu), diagnosed on lymph node biopsy, that included biopsies of involved extranodal sites [bone marrow (5), tonsil (1), and stomach (1)], 2 cases of enteropathy-type T-cell lymphoma (ETTL), and 1 case of extranodal NK/T lymphoma, nasal type were included as controls.

Immunohistochemistry

Paraffin sections (3 μm) were immunostained by the streptavidin immunoperoxidase method (ChemMate Streptavidin Peroxidase kit, Dako, Cambridge, United Kingdom) and DAB chromogen (Dako) following heat-mediated antigen retrieval as previously described.[5] Primary antibodies included CD3 (polyclonal anti-CD3; Dako), CD10 (56C6, Novocastra Labs, Newcastle, United Kingdom), CD20 (L26; Dako), and CD21 (1F8; Dako). Sequential double staining using CD20/CD10 was done on selected cases, in each case the first antibody being revealed with peroxidase and the second with alkaline phosphatase with a fast blue chromogen in each case.[13] No counterstain was used. To demonstrate CD10 expression in neoplastic T cells, CD10, and CD20/CD10 sections were compared with sequential CD3 immunostained sections as described previously.[1]

TABLE 1. Clinical Features, Site of Biopsy, and Initial Diagnosis

Case No.	Age (yr)/Sex	Clinical Presentation	Site of Biopsy	Initial Diagnosis
1	33/F	Not available	Lymph node Tonsil Cecum (1 year after diagnosis)	AITL Not available Recurrent lymphoma or early inflammatory bowel disease
2	75/F	Shortness of breath and generalized lymphadenopathy; chest X-ray showed an expanding discrete lesion in the right upper lobe; 2 years later she re-presented with malaise, weight loss, hepatosplenomegaly, and lymphadenopathy	Lung Inguinal lymph node (2 years after lung biopsy) Bone marrow	Reactive lymphoid hyperplasia Hodgkin lymphoma Staging-?involved
3	82/M	B-symptoms, anemia, generalized lymphadenopathy, splenomegaly.	Lymph node Bone marrow	Reactive-?connective tissue disorder Staging-? involved
4	43/F	Lymphadenopathy 2 years later, presented with pneumonitis	Lymph node Lung	AITL ? involved by AITL
5	48/M	Not available	Lymph node Bone marrow	AITL Staging-? involved
6	63/F	Presented with pulmonary embolism and subsequently shown to have splenomegaly and cervical and abdominal lymphadenopathy	Cervical lymph node Bone marrow	AITL or PTL Staging-? involved
7	67/M	Bilateral axillary and inguinal lymphadenopathy	Inguinal lymph node Bone marrow	High-grade NHL Staging-? involved
8	58/M	Generalized lymphadenopathy and skin rash	Axillary lymph node Bone marrow	AITL Staging-involved
9	81/F	Not available	Cervical lymph node Nasopharynx	NHL NHL
10	59/M	Large mass in postnasal space and multiple enlarged cervical lymph nodes	Cervical lymph node Nasopharynx	Lennert's lymphoma Lennert's lymphoma

AITL, angioimmunoblastic T-cell lymphoma; PTL, peripheral T-cell lymphoma; NHL, non-Hodgkin's lymphoma.

TABLE 2. Summary of Histology, Immunophenotypic Profile, and T-Cell Clonality Analysis of Involved Extranodal Biopsies

Case No.	Extranodal Site	Histology			Immunohistochemistry		TCR PCR
		Follicles	Vascularity	Clear Cells	CD21+ FDC Meshwork	CD10+ T Cells	
1	Tonsil	Occasional regressed	Rich network	Present	Expanded with sprouts encircling vessels	Numerous	Not done
	Cecum	No identifiable follicles	Rich network	Present	Expanded with sprouts encircling vessels	Numerous	Oligoclonal
2	Lung	Hyperplastic	Interfollicular increase	Absent	Highlights follicles; one focus expanded with sprouts	Many	Monoclonal (same size band as lymph node)
	Bone marrow	No identifiable follicles	Increased	Absent	Absent	Absent	Monoclonal (same size band as lymph node and lung)
3	Bone marrow	Regressed follicles	No increase	Absent	Highlights regressed follicles	Present	Poor DNA
4	Lung	No identifiable follicles	Increased	Present	Highlights follicles; no sprouts	Present	Monoclonal (same size band as lymph node)
5	Bone marrow	No identifiable follicles	No increase	Absent	Absent	Absent	Poor DNA
6	Bone marrow	No identifiable follicles	No increase	Absent	Absent	Absent	Monoclonal (same size band as lymph node)
7	Bone marrow	No identifiable follicles	No increase	Absent	Absent	Absent	Monoclonal (same size band as lymph node)
8	Bone marrow	No identifiable follicles	Increased	Absent	Absent	Absent	Not done
9	Nasopharyngeal biopsy	Hyperplastic	Interfollicular increase	Absent	Highlights follicles	Present	Inconclusive
10	Nasopharyngeal biopsy	No identifiable follicles	Increased	Absent	Expanded with sprouts encircling vessels	Present	Monoclonal

FDC, follicular dendritic cells; TCR, T-cell receptor-γ chain gene; PCR, polymerase chain reaction.

In Situ Hybridization for Epstein Barr Virus (EBV)–Epstein Barr Early Region (EBER)

In situ hybridization (ISH) was carried out with a polymerase chain reaction (PCR)-generated EBV DNA probe labeled with digoxigenin, followed by incubation with antidigoxigenin-AP (Boehringer Mannheim, Germany) and visualization with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium as previously described.[7]

PCR for T-cell Receptor-γ (TCR-γ) Chain Gene

PCR was performed to analyze clonal expansion of T cells. DNA was extracted from paraffin sections using protein-

ase K digestion without subsequent organic extraction as previously described.[13] T-cell clonal expansion was detected by analysis of TCR-γ chain gene rearrangement. Duplicate aliquots of each sample were analyzed for rearrangement using two sets of primers as previously described.[1] DNA extracted from a paraffin block of a T-cell lymphoma was used as a positive control, and a reaction without template DNA was run as a negative control in all experiments. PCR products were analyzed on 10% polyacrylamide minigels, stained with ethidium bromide, and viewed under UV light.

RESULTS

Clinical Features

The clinical presentations and the initial diagnoses at referral are summarized in Table 1. Among the 10 cases studied, there were 5 females and 5 males between the ages of 33 and 82 years. In 5 of the 7 cases for whom clinical history was available, systemic symptoms were prevalent at some time during the course of the disease. Six cases had involved bone marrow trephines, 2 had pulmonary involvement, 1 had tonsillar and cecal involvement, while 2 cases had involved nasopharyngeal biopsies. Lymph node biopsies were performed for initial diagnosis in 9 of the 10 cases, while in 1 case (case no. 2) a lung biopsy was followed 2 years later by a lymph node biopsy. An initial diagnosis of AITL was made in only 5 of the 10 cases.

Histology

Lymph Nodes

Angioimmunoblastic T-cell Lymphoma

Lymph node biopsies in cases nos. 1-8 and 10 conformed to that described as typical for AITL, with effacement of architecture by a polymorphic infiltrate comprising small lymphocytes and transformed blasts, plasma cells, histiocytes, and eosinophils within a prominent vascular network. A proportion of the lymphoid blasts in case nos. 1, 2, and 4 had pale to clear cytoplasm. Follicles were either not identifiable or regressed with concentrically arranged FDC. The pattern of involvement in case no. 9 was similar to that described in early AITL,[1] with preserved hyperplastic follicles, and a paracortical infiltrate similar to that seen in the other cases.

Extranodal Sites

Angioimmunoblastic T-cell Lymphoma

The histologic features of extranodal sites of involvement of each case are summarized in Table 2. The features ranged from a nonspecific mixed infiltrate (bone marrow biopsies in case nos. 5, 6, and 7) to a polymorphic infiltrate with clear cells in close proximity to a prominent arborizing vascular network (tonsillar and cecal biopsies in case no. 1; Fig. 1A,D,G, J). All 6 involved bone marrow trephines showed focal involvement, which was paratrabecular in 4 cases. Two cases showed an increase in vascularity (Fig. 1J). Three involved trephines (case nos. 5, 6, and 7) showed a mixed infiltrate of small and large lymphoid cells. One case (case no. 3) had evidence of regressed follicles in close association with the neoplastic infiltrate, while the 2 biopsies with increased vascularity (case nos. 2 and 8) comprised foci with small and large lymphocytes, epithelioid histocytes, fibroblasts, and eosinophils amid the vessels imparting a "granulomatous" appearance (Fig. 1J).

Immunohistochemistry

Lymph Nodes

Angioimmunoblastic T-cell Lymphoma

The infiltrate comprised a predominance of CD3-positive T cells. In case nos. 1-8 and 10, CD21 highlighted the hyperplastic FDC meshwork that extended into the paracortex to surround high endothelial venules. In case no. 9, CD21 immunostaining was more or less confined to the hyperplastic follicles identified on histology, with only subtle extensions into the paracortex, consistent with early AITL.[1] Single layered immunohistochemistry with CD10 and double staining with CD20/CD10 highlighted a population of CD10-positive, CD20-negative lymphoid cells in 8 cases (case nos. 3-10). Examination of sequential sections immunostained for CD3 and CD20/CD10 showed that these CD10-positive cells were the neoplastic T cells, as previously reported. The distribution of these cells was similar to that of the expanded FDC meshwork. In case no. 9, which had hyperplastic follicles, the CD10-positive T cells were situated at the outer rim of the follicle spilling into the paracortex. No CD10-positive T cells were identified in the lymph node biopsy in case no. 2. CD10 expression could not be investigated in the lymph node biopsy in case no. 1 as insufficient material was available.

Peripheral T-cell Lymphoma, Unspecified

No CD10-positive lymphoid cells were present in any of the cases of PTLu.

Extranodal Sites

Angioimmunoblastic T-cell Lymphoma

CD3 immunostaining highlighted a marked T-cell infiltrate in all cases. The details of CD21 expression and CD10 expressing T cells are given in Table 2. At one end of the spectrum, CD21 immunostaining showed mild expansion of the FDC meshwork as in the tonsillar and cecal biopsies of case no. 1 (Fig. 1B, E). Focally, the FDC surrounded the venules in these biopsies. FDC expansion was subtle but nevertheless present focally, in the lung biopsy of case no. 2 (Fig. 1H). At the other extreme, there were no CD21-positive FDC mesh-

works in 5 of the 6 bone marrow biopsies (Fig. 1K). In all other biopsies, the FDC meshwork highlighted either hyperplastic or regressed follicles but showed no expansion (Fig. 1N). CD10 and CD20/CD10 double immunostaining highlighted CD10-positive, CD20-negative lymphoid cells corresponding to T cells in all sites except in the bone marrow (Fig. 1C, F, I). In the bone marrow, the presence of non-neoplastic hematopoietic precursors and the subtle nature of involvement made assessment of CD10 positivity of the neoplastic cells more difficult. Nevertheless, after careful comparison with morphology and CD3 immunostaining, aberrant CD10 expression by neoplastic T cells could be identified only in one involved bone marrow trephine (Fig. 1L, O). Aberrant CD10 expression in all involved extranodal sites correlated well with the presence and distribution of FDC (Fig. 1). In those cases in which the FDC showed minimal expansion or sprouting (lung biopsies in case nos. 2 and 4, bone marrow biopsy in case no. 3, and nasopharyngeal biopsy in case no. 9), CD10-positive T cells were seen at the edge of the follicle spilling into the adjacent interfollicular region (Fig. 1O).

Other Peripheral T-cell Lymphomas

No CD10-positive lymphoid cells were present in any of the cases of PTLu. The neoplastic cells in both cases of ETTL and in the case of extranodal NK/T lymphoma, nasal type were CD10 negative.

In Situ Hybridization for EBV-EBER

EBER-ISH was performed on lymph nodes in case nos. 2-10. Lymph nodes in case nos. 2-8 and 10 showed hybridization for EBV-EBER in a subset of cells that appeared to correspond to CD20-positive B-blasts. Case no. 9 was EBER-ISH negative. EBER-ISH performed on the cecum in case no. 1 and the nasopharyngeal biopsy in case no. 9 was negative.

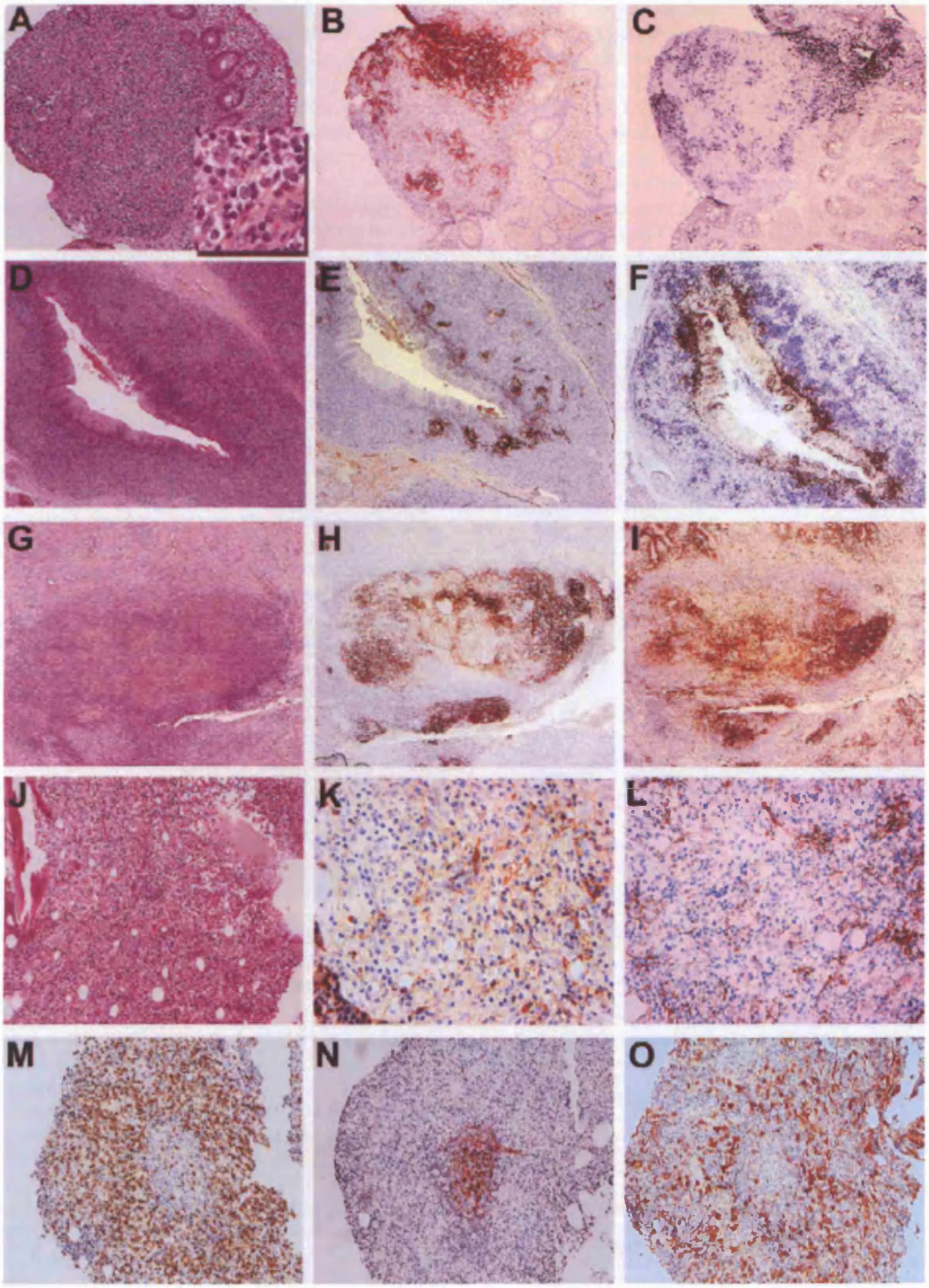
PCR for TCR-γ Chain Gene

The results of PCR for TCR-γ chain gene in the 10 cases of AITL are shown in Table 2. All but one case with good quality DNA showed either a single or two distinct bands, consistent with monoclonal T-cell expansion. Case no. 1 showed 3 bands and was interpreted as being biclonal/oligoclonal.

DISCUSSION

The diagnosis of AITL is based on histologic examination of a lymph node and requires demonstration of architectural, cytologic, and immunophenotypic changes often combined with molecular genetic analysis.[8,11] Of the morphologic features described as being typical for AITL, a polymorphic infiltrate and prominent high endothelial venules are rather nonspecific, and shared by reactive conditions and other lymphomas. Clear cells, considered characteristic, are present in less than half the cases.[1] In practice, it is the proliferation of FDC, best appreciated by immunohistochemistry, and its association with vessels, that is most specific, and ultimately helps to distinguish AITL from PTLu.[11,14] However, it is now known that, in early cases, this pattern of FDC hyperplasia may be subtle if present at all, and follicular hyperplasia, once thought to exclude AITL, is a prominent feature.[1,19] Thus, except when changes are florid, specific histologic diagnosis can be difficult, even on lymph node biopsy, requiring correlation with immunohistochemistry, molecular genetics, and importantly, the clinical presentation.[1,4] This difficulty also questions the diagnostic accuracy of early reports of extranodal involvement, when AITL was thought to be an atypical reactive process, rather than a lymphoma, and diagnosis was purely on morphologic grounds, with no resort to immunohistochemistry or molecular genetic evidence of T-cell clonality. Nevertheless, in both early and more recent reports of visceral involvement by AITL, the histologic features range from a polymorphous infiltrate with prominent vascularity and interstitial periodic acid-Schiff positive material to follicular hyperplasia with an interfollicular polymorphous infiltrate, similar to that observed in early lymph node involvement.[10,15,16,20,23 25] In our study, the histology in involved extranodal sites such as lung, nasopharynx, cecum, and tonsil showed a similar spectrum of histologic changes, ranging from those that may be mistaken for a reactive lymphoid proliferation to features sug-

FIGURE 1. Histology, follicular dendritic cell meshwork, and CD10 expression in extranodal biopsies of case nos. 1-3. **A-F:** The cecal (panels A-C) and tonsillar biopsies (panels D-F) from case no. 1, panels **G-L**, the lung (panels G-I) and bone marrow biopsies (panels J-L) from case no. 2, and panels **M-O** the bone marrow biopsy from case no. 3. A, D, G, J: Hematoxylin and eosin-stained sections of case nos. 1 and 2 show increased vascularity and a polymorphous infiltrate. The inset in A shows atypical clear cells in the vicinity of a vessel. J: The bone marrow biopsy from case no. 2 shows a "granulomatous" appearance. The inset in A highlights the polymorphous nature of the infiltrate and the presence of clear cells in close proximity to vessels. M: CD3 shows a prominent T-cell infiltrate in the bone marrow of case no. 3. B, E, H, K, N: Immunostaining for CD21 in case nos. 1-3. There is mild expansion of follicular dendritic cell meshwork (FDC) in the cecal (B) and tonsillar biopsies (E) in case no. 1 and focal expansion in the lung biopsy (H) in case no. 2, but no evidence of FDC in the bone marrow biopsy (K) of case no. 2. The bone marrow biopsy in case no. 3 (N) shows a regressed follicle. C, F, I, L, and O: CD10 immunostaining in case nos. 1-3. C and F: Double immunostaining for CD20 in brown (DAB) and CD10 in blue (fast blue) highlight numerous CD20-negative, CD10-positive cells, consistent with T cells. Single layer CD10 immunostaining (I, L, and O) shows many CD10-positive lymphoid cells in the bone marrow biopsy in case no. 3 (O) and in the lung biopsy (I), but not in the bone marrow biopsy (L) of case no. 2. (Original magnifications: A-I, X 40; J, M-O, X 100; K, L, A inset, X 1000.)



gestive of lymphoma, but not specifically AITL. A specific phenotypic or molecular marker would thus be the only means of definite diagnosis of AITL at these sites, in the absence of an accompanying lymph node biopsy. In our study, aberrant expression of CD10, a feature shown to be a sensitive and specific marker of neoplastic cells in affected lymph nodes, was consistently seen in all involved extranodal sites, except 5 of 6 involved bone marrow trephines. Its value is especially highlighted in case no. 2 in which the misdiagnosis of reactive (follicular) hyperplasia on lung biopsy may have been averted if aberrant CD10 expression had been assessed. Although sequential double immunostaining using CD20/CD10 is useful to demonstrate aberrant CD10 expression by the neoplastic T cells, comparison of consecutive sections with routinely performed single layer immunohistochemistry using CD20, CD3, and CD10 would have sufficed in many cases.

Bone marrow is biopsied as a part of the staging procedure and is often involved at diagnosis.[3,10] In their series of 8 cases, Ghani and Krause described focal infiltrates with prominent epithelioid cells imparting a "granulomatoid appearance" as the predominant pattern of involvement.[10] In the same study, paratrabecular involvement described as typical for AITL by Pangalis et al[17] was a rare feature, and an increase in vascularity, prominent in involved lymph nodes, was seen in only 3 of their cases.[10] In our study, although focal involvement was seen in all involved bone marrow biopsies, two thirds (4 of 6 cases) of which showed a paratrabecular distribution, a "granulomatoid" appearance was seen only in one third of the cases (2 of 6 cases). An increase in vascularity was also observed only in the latter 2 cases. FDC, a prominent feature in involved lymph nodes, was observed only in case no. 3 in the form of regressed follicles. In all 6 cases, the morphologic, immunophenotypic, and molecular genetic features on bone marrow trephine enabled a diagnosis of peripheral T-cell lymphoma but were not sufficient to subtype further as AITL. Aberrant CD10 expression, a useful feature in this situation, was however present only in one case (case no. 3).

Skin rash is a common symptom at presentation.[22] Unfortunately, because of the lack of involved skin biopsies in our series, we were unable to assess the usefulness of aberrant CD10 expression at this site.

CD10, a transmembrane protein with neutral endopeptidase activity, is expressed in follicle center B cells and is a reliable marker of follicular lymphoma.[5] In the latter, CD10, although strongly expressed within the neoplastic follicles, is down-regulated in clonally identical interfollicular neoplastic cells.[6] The expression of CD10 in follicular lymphoma cells may thus be dependent on a signal from the FDC. In vitro studies have shown that highly purified FDCs are able to induce the proliferation of allogeneic T cells or T-cell lines.[2] There is, however, no direct evidence for ongoing interactions between the neoplastic cells of T-cell lymphomas and FDC. Nevertheless, AITL is characterized by an expansion of FDC meshwork.[11] In lymph nodes and extranodal sites, aberrant CD10 expression correlates well with the presence and distribution of FDC. This correlation is especially well highlighted in the cases with bone marrow involvement in our study, where aberrant CD10 expression in the marrow is confined to the only case associated with FDC. Conversely, in case nos. 2 and 5-8, the neoplastic cells are CD10 negative in the marrow when devoid of associated FDC, but CD10 positive in other involved sites that harboured FDC. This suggests an analogy with follicular lymphoma where, despite clonal identity, there is down-regulation of CD10 in neoplastic cells that lack association with FDC and indicates a possible role of FDC in this phenomenon. In support of this theory, Kim et al have shown in a recent study that FDC and endothelial cells in AITL express Fas ligand (FasL) whereas the CD10-expressing tumor T cells express Fas (CD95) and caspase 3, indicating Fas-FasL interaction between the neoplastic T cells and the FDC and suggest that the follicular milieu is necessary for CD10 expression by tumor cells, a feature that may play a functional role in regulating apoptosis.[12]

In summary, aberrant expression of CD10 by neoplastic T cells in AITL is maintained in most involved extranodal sites and shows good correlation with the presence and distribution of FDC. This immunophenotypic feature may thus be used to make a diagnosis of AITL in an extranodal site, even in the absence of accompanying lymph node histology.

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